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TITLE: Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance

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14. ABSTRACT

During Year 3, revisions were made to the protocol, informed consent, and HIPAA documents to allow for additional data collection during the hospital portion of the study via actigraphy. Recruitment materials were also revised in order to target a non-smoking population. This greatly reduced the amount of participants ruled out during the phone screening process, improving phone screening success. Safety reports continue to be sent to Actelion on a monthly basis. Both blinded and unblinded monitoring visits of study procedures and facilities are ongoing. Throughout Year 3, many new study team members were hired and trained (Study Coordinator, Recruiter, Research Assistant, and Lab Manager). The study team also expanded to include additional informed consent administrators, clinical interviewers, as well as sleep technicians and neuropsychological assessment administrators in order to reach the new quarterly enrollment goal of 39 participants. Enrollment is expected to increase throughout Year 4 due to the actions taken above.

15. SUBJECT TERMS
Neurocognitive Performance, Sleep, Hypocretin, Orexin

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ANNUAL PROGRESS REPORT
September 28, 2012

Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance
USAMRMC Grant W81XWH-09-2-0080
Thomas Neylan, M.D., Principal Investigator

INTRODUCTION

An integrated translational study will be conducted to examine the effect of a novel hypocretin/orexin antagonist, almorexant (ALM), compared to a standard hypnotic, zolpidem (ZOL), and placebo (PBO) on neurocognitive performance at peak concentration post dosing. The human study component (Task 1; responsible individual: Thomas Neylan, M.D.) will establish whether ALM is superior to ZOL in relation to neurocognitive side effects. It is hypothesized that healthy human subjects receiving zolpidem 10mg will show greater impairment in neurocognitive performance compared to subjects receiving 100mg or 200mg doses of almorexant or placebo. Study subjects (n=200) will receive a randomly assigned, one-time dose of study drug in an inpatient hospital setting. A battery of neurocognitive, objective alertness, and subjective symptom assessments will be administered prior to and following dosing. Assessments to be administered were selected based upon their demonstrated sensitivity to sleep-inducing agents and their military relevance. The animal study component (Tasks 2 – 5; responsible individual: Thomas Kilduff, Ph.D.) will compare the neural circuitry that underlies the activity of the abovementioned compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Progress associated with each task outlined in the approved Statement of Work is listed below:

Task 1: *Test the hypothesis that healthy human subjects receiving ZOL 10mg will show greater impairment in neurocognitive performance compared to subjects receiving PBO or the 2 doses (100mg, 200mg) of ALM.*

The Task 1 subtasks listed below have been completed prior to or during Year 3:

Subtask #1: Write Protocol

The study protocol was finalized during Year 1, and modifications were made to the protocol during Year 2 and Year 3. The current version of the protocol is included in Appendix 1.

Subtask #2: Obtain Scientific and Human Use Approvals

Study documentation was submitted to the appropriate Institutional Review Boards (IRBs) and the Food and Drug Administration (FDA) for approval prior to the end of Year 1. All human subjects approvals were obtained during Year 2. Approval timelines are detailed below:

- IRB Approval:

  Initial Approval

  The University of California, San Francisco Committee on Human Research (UCSF CHR) provided initial approval on October 29, 2010. The Department of Veterans Affairs Medical Center Research and Development Committee (VA R&D Committee) provided approval on January 6, 2011. The U.S. Army Medical Research and Materiel Command Human Research Protection Office (USAMRMC HRPO) provided initial approval on March 9, 2011.

  Approval of Amendments

  An amended Investigator's Brochure was provided by Actelion Pharmaceuticals on March 23, 2011 which necessitated revisions to the study protocol and informed consent document. Enrollment could not begin until all Institutional Review Boards approved the revised study documents. The UCSF CHR and the VA R&D Committee approved the revisions on May 3, 2011. The USAMRMC HRPO approved the revisions on May 10, 2011, at which point enrollment could be initiated. Informed consent and VA HIPAA documents were revised and approved by UCSF CHR on February 7, 2012 (modifications included giving participants the option to consent to be contacted for participation in other research studies within the Stress & Health Research Program, as well as requesting that participants continue filling out a sleep diary and wearing an actigraph during the hospital portion of the study).

  Continuing Review

  An annual continuing review application was approved by the UCSF CHR on September 19, 2012, extending the study's approval expiration to October 4, 2013. Continuing review approval from the VA R&D Committee was received on September 25, 2012. All continuing review approvals were submitted to a continuing review analyst at the USAMRMC HRPO on September 27, 2012.

- Investigational New Drug Application (IND): At the conclusion of Year 1, an IND application was filed with the FDA in order to obtain approval to receive study drug from Actelion Pharmaceuticals. The IND went into effect on October 21, 2010. The study protocol was re-submitted to the FDA on May 18, 2011 following revisions in response to the Investigator's Brochure Amendment received from Actelion Pharmaceuticals in March, 2011. In accordance with FDA requirements, an annual progress report will be submitted by no later than December 20, 2012.

Subtask #3: Purchase Study Related Equipment/Supplies

The majority of study related equipment (including sleep equipment, actigraphs, psychomotor vigilance tests, and neuropsychological testing supplies) was purchased and tested during Year 1. Further testing and piloting of the equipment was performed during the early part of Year 2. All remaining study supplies (including drug testing kits and additional sleep equipment) were purchased and tested during Year 2.

Study drug (provided by Actelion Pharmaceuticals) arrived onsite at the UCSF Medical Center pharmacy in March, 2011. An external unblinded monitor has been appointed to perform regular

drug accountability checks to confirm that drug is stored properly and in accordance with expiration dates. The most recent unblinded monitoring visit took place on June 20, 2012.

Clinical Trial Management Software was purchased during Year 3, but this software will not be used for study 10-02811 because the anticipated benefits of the software do not currently outweigh the time and labor needed to transfer data from the current database.

Subtask #4: Train Laboratory Personnel

Key study personnel were hired and trained during Year 1 and Year 2. During Y3, Q1, the original Recruiter left the team and a new Recruiter was hired and trained. During Y3, Q2, the Study Coordinator left the team, and the original Research Assistant assumed the position of Study Coordinator. A new Research Assistant was hired and trained shortly thereafter. In addition, the Project Manager left the team and a new Lab Manager was hired. The responsibilities of the Lab Manager (solely in respect to study 10-02811) are listed below:

- Lab Manager: The Lab Manager oversees the Study Coordinator and provides guidance with the submission of all regulatory documents to UCSF CHR, the VA R&D Committee, and USAMRMC HRPO. She also provides support for a variety of other study procedures such as discharging study participants from UCSF.

Lastly, Anne Richards was added to the protocol as an additional investigator during Y3, Q2.

Subtask #5: Collect Data on 200 Volunteers

Recruitment and enrollment efforts were initiated in May 2011 (Y2, Q3), following receipt of all regulatory approvals. Enrollment details and future plans are outlined below:

A.) Enrollment Progress During Year 3

- **Advertising**: Advertising efforts have involved monthly postings on the internet. These ads have generated a substantial response rate, as approximately 920 individuals have shown an interest in the study throughout the past year. Throughout Year 3, 1,002 interested participants were screened by phone prior to being scheduled for full eligibility assessments.
- **Screening**: During Year 3, 102 participants met phone screen requirements, provided informed consent, and were invited to take part in full screening procedures at the San Francisco VA Medical Center. Screening procedures include a mental health screening, self-report questionnaires related to caffeine use, tobacco use, alcohol use, and sleep habits, a physical exam, urine drug and pregnancy screen, and blood draw for hematology and serum chemistry panels.
- **Eligible Participants**: Throughout Year 3, 47 participants were identified as eligible. 33 of those participants have completed all study procedures and 2 participants were dosed but lost to follow-up.

B.) Enrollment/Recruitment Challenges Faced During Year 3:

- **Enrollment Challenges**
  Enrollment slowed during Y3, Q2 during the transition to a new Study Coordinator in January 2012. Once the new Study Coordinator was trained, eligibility procedures and

enrollment greatly improved. Whereas only 14 participants were consented in Y3, Q2, 32 participants were consented in Q3, and 42 participants were consented in Q4. Three additional staff members were also trained in administering informed consent in order to increase screening efficiency and boost enrollment.

- **Recruitment Challenges**
  Recruitment efforts slowed in Y3, Q1 and Q2 while a new Recruiter was hired and trained. Whereas only 85 potential participants were phone screened during Y3, Q2, recruitment procedures significantly improved throughout Q3 (288 potential participants were phone screened) and Q4 (266 potential participants were phone screened). Recruitment metrics suggest that for the first six months of the year, only 18% of potential participants phone screened were deemed eligible to come in for an informed consent meeting. The majority of rule outs that occurred during the phone screening process was due to tobacco use (23%), and so new recruitment text was created to target a non-smoking population. The percentage of rule outs due to tobacco use decreased from 23% to only 4%. Moreover, screening success virtually doubled by the end of Y3, Q4—32% of potential participants phone screened were deemed eligible and invited to come in for an informed consent meeting versus the former 18% success rate.

C.) Future Enrollment Strategies:

- **Recruitment and Outreach**:
  Revised recruitment materials were recently submitted to the UCSF CHR and were approved on June 13, 2012. The revised recruitment materials emphasize that healthy volunteers are needed for the study and that participants must be non-smokers and maintain a healthy weight. It is anticipated that this will continue to decrease the amount of participants who are ruled out for tobacco and marijuana use as well as BMI outside of our required study range. Outreach efforts planned for the immediate future include the continued distribution of posters, flyers, and postcards on the campuses of local universities. The recruitment team also plans to look into utilizing various forms of social media (such as social networking sites) in order to target the intended population.

- **Monthly Enrollment Projections:**
  By the end of Year 3, a total of 43 participants completed all study procedures. In order to enroll 200 participants by the end of Q2, Y4, the quarterly enrollment target of 33 participants will need to increase to 39 quarterly participants, or 13 participants per month. The study team is taking a variety of steps to reach this goal including increasing study staff (e.g. informed consenters, neuropsychological assessment administrators, and clinical interviewers) and improving recruitment materials to target common reasons for rule outs during the phone screening process.

Subtask #6: Score and Analyze Data

Study data has been scored and cleaned on an ongoing basis since the initiation of enrollment to shorten the cleaning and analysis timelines required during Y4. All data is QC'd and scored by trained and qualified study staff. Data Entry is ongoing and up to date.

Other Accomplishments Completed During Year 3:

- Reporting:
  Ongoing reports have been submitted as follows:
  - Safety listings are submitted to Actelion Pharmaceuticals on a monthly basis.
  - Progress reports are submitted to the Department of Defense on a quarterly basis.
  - Progress reports are submitted to the FDA on an annual basis.

- Human and Animal Study Collaboration:
  The San Francisco (human study) and SRI International (animal study) teams met monthly via teleconference throughout Year 3 to share progress updates, scientific rationale, and future planning initiatives. An in-person collaborative meeting was hosted by SRI in August 2011 and the most recent meeting was hosted at the SFVAMC in September 2012, just after the conclusion of Year 3. At each in-person meeting, members from each team gave presentations related to research rationale, progress, and future directions.

Tasks 2 – 5: Please refer to the attached report from Dr. Kilduff (Appendix 2) which details the progress made in reference to the animal studies.

KEY RESEARCH ACCOMPLISHMENTS

Task 1 Accomplishments:

- Revised informed consent and HIPAA documents have been approved by UCSF CHR.
- All new study personnel (Study Coordinator, Recruiter, and Research Assistant) have been hired and trained on the study protocol and procedures.
- 47 eligible participants have been identified through recruitment and screening efforts.
- Recruitment materials have been revised to increase phone screening success and boost enrollment—screening success has already doubled since the beginning of 2012.

Tasks 2 – 5 Accomplishments:
Please refer to the attached progress report from Dr. Kilduff (Appendix 2).

REPORTABLE OUTCOMES

Reportable outcomes related to Task 1 will not be available until Year 4. Reportable outcomes related to Tasks 2 – 5 are noted in the attached progress report from Dr. Kilduff (Appendix 2).

CONCLUSION

Preclinical data indicate that animals treated with almorexant are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The purpose of this research is to test related hypotheses in both animals and humans. Enrollment of human subjects began during Year 2 and is expected to continue until the middle of Year 4. The Year 2 findings from the animal component of the study were

consistent with the hypothesis that disfacilitation of wake-promoting systems by almorexant results in less functional impairment than the general inhibition of neural activity produced by zolpidem (Appendix 2).

APPENDICES

Appendix 1: Human Study Protocol

Appendix 2: Animal Studies Progress Report

Appendix 1: Human Study Protocol

CLINICAL STUDY PROTOCOL

Title:	Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance
Protocol number:	NEY-1413
Protocol Version/Date:	Final Version 8.0 05 January 2012
Phase:	Investigator-Initiated
Investigational Drug:	Almorexant
Investigator-Sponsor:	Thomas C. Neylan, M.D. Northern California Institute for Research and Education 4150 Clement Street (116P) San Francisco, CA 94121
Medical Monitor:	Frank Schoenfeld, MD Northern California Institute for Research and Education 4150 Clement Street (116P) San Francisco, CA 94121
Study Sites:	University of California, San Francisco Clinical and Translational Sciences Institute Clinical Research Center 505 Parnassus Avenue San Francisco, CA 94143 San Francisco Department of Veterans Affairs Medical Center 4150 Clement Street San Francisco, CA 94121

This clinical study will be conducted in accordance with Standard Operating Procedures (SOPs), current Good Clinical Practice (GCP) and the provisions of International Conference on Harmonization (ICH) Guidelines

Protocol Approval
NEY-1413
Final Version 8.0 05 January 2012

Investigator-Sponsor		
	Thomas Neylan, MD	**Date**
Co-Investigator		
	Steven Batki, MD	**Date**
Co-Investigator		
	Kristin Samuelson, Ph.D.	**Date**
Biostatistician		
	Thomas Metzler, M.S.	**Date**
Study Coordinator		
	Mindy Sivasubramanian, M.S.	**Date**

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ABBREVIATIONS

AE	Adverse Event
AASM	American Academy of Sleep Medicine
BzRAs	Benzodiazepine Receptor Agonists
CRC	Clinical Research Center
CCRC	University of California, San Francisco Clinical Translational and Sciences Institute Inpatient Clinical Research Center
UCSF CHR	University of California, San Francisco Committee on Human Research
CNS	Central Nervous System
CPT	Conners' Continuous Performance Test II
CRF	Case Report Form
DMP	Data Management Plan
DS	Digit Span Subtest of the Wechsler Adult Intelligence Scale Fourth Edition
DSM-IV-TR	Diagnostic and Statistical Manual of Mental Disorders Fourth Edition Text Revision
EEG	Electroencephalogram
FDA	Food and Drug Administration
GABA	Gamma-Aminobutyric Acid
GCP	Good Clinical Practice
GP	Grooved Pegboard Motor Test
ICH	International Conference on Harmonisation
IND	Investigational New Drug Application
IQ	Intelligence Quotient
IRB	Institutional Review Board
MWT	Maintenance of Wakefulness Test
NREM	Non-Rapid Eye Movement
ORP HRPO	Federal Office of Research Protections Human Research Protection Office
P-A	Paired Associates Learning Task
PSG	Polysomnography
PSQI	Pittsburgh Sleep Quality Index
PVT	Psychomotor Vigilance Test
QC	Quality Control
R&D Committee	Veterans Affairs Research and Development Committee

REM	Rapid Eye Movement
RAVLT	Rey Auditory Verbal Learning Test
SAE	Serious Adverse Event
SC	Symptom Checklist
SCID	Structured Clinical Interview for DSM-IV TR Axis I Disorders
SFDVAMC	San Francisco Department of Veterans Affairs Medical Center
SSS	Stanford Sleepiness Scale
Stroop	Stroop Color-Word Test
Towers	Tower Test from Delis-Kaplan Executive Function System
USAMRMC	U.S. Army Medical Research Materiel Command
WAIS-IV	Wechsler Adult Intelligence Scale Fourth Edition
WASO	Wake after Sleep Onset
WMS	Wechsler Memory Scale

SYNOPSIS

Protocol Number:	NEY-1413
Study Title:	A Double-Blind, Placebo-Controlled, Randomized, Parallel-Group Study Comparing the Effect of a Novel Hypocretin/Orexin Antagonist (Almorexant) Versus a Standard Hypnotic (Zolpidem) and Placebo on Neurocognitive Performance
Number of Sites:	1
Treatment Duration:	One-time Dose
Study Duration:	10 days, with a follow-up visit within 5 – 12 days of dosing
Study Population:	216 healthy male and female volunteers
Rationale:	In recent years, there has been increased focus on neurocognitive effects of hypnotic medications that adversely affect behavior during unanticipated awakenings during the night. Concerns regarding untoward effects of hypnotics during the sleep period have led to a Food and Drug Administration (FDA) class warning for all hypnotic drugs. These concerns are particularly relevant to the personnel of the military and those in other professions who have an occupational risk of poor sleep and who are expected to perform without impairment upon awakening. Almorexant is a hypocretin/orexin antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with almorexant are easily aroused from sleep and behave free of ataxia and other impairment. If this observation is confirmed in humans, it will have substantial implications for the management of disturbed sleep in both military and civilian populations.
Study Objectives:	To compare neurocognitive performance at peak concentration at midpoint during the habitual wake period in subjects randomized to almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo.
Study Design:	The study will take place at the San Francisco Department of Veterans Affairs Medical Center (SFDVAMC) and the University of California, San Francisco Clinical Translational and Sciences Institute inpatient Clinical Research Center (CCRC). The study will involve healthy volunteers who are considered normal sleepers per the Research Diagnostic Criteria for Normal Sleepers and who are free of medical disorders and specified psychiatric disorders. After informed consent has been obtained and eligibility has been

	confirmed, subjects will be scheduled for the 10-day study period. During the first seven days of the study period (the sleep/wake monitoring period), subjects will be asked to maintain a sleep diary and wear a wrist activity monitor (actigraph) 24 hours per day. Subjects will be admitted to the CCRC on the eighth day of the study period, two days prior to study drug administration. Subjects' sleep will be monitored with polysomnography (PSG) during each night on the CCRC, and subjects will continue to maintain a sleep diary and wear an actigraph during the three-day hospital stay. Subjects will be randomized in a double-blind fashion to one of four groups (almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo). Study drug will be provided to a nurse on the CCRC by an unblinded research pharmacist. The nurse and all other study personnel will remain blinded when study drug is dispensed to subjects. Following dosing, subjects will be accompanied by study personnel and instructed to remain awake. Neurocognitive, objective alertness, and subjective symptom assessments will be administered for several hours following dosing. Adverse events (AEs) will be assessed at the time of admission to the CCRC and on each day of the subject's stay in the CCRC. Subjects will be debriefed and discharged from CCRC on the morning of the fourth day on the unit. They will be required to return to the CRC at the SFDVAMC within 5 – 12 days of dosing for a safety lab test (liver function).
Inclusion Criteria:	1.) Male and female subjects between the ages of 19 and 39 determined to be physically healthy by physical exam and laboratory assessments; 2.) Habitual wake time between 0600 hr and 0900 hr maintained within the past month; 3.) Habitual bedtime between 2200 hr and 0100 hr maintained within the past month; 4.) Body Mass Index (BMI) >18 and < 28 kg/m^2; 5.) Ability to communicate well with the Investigator and to understand the study requirements.
Exclusion Criteria:	1.) Diagnosis of a sleep disorder within two years of screening or current sleep disturbance as suggested by a global score of > 5 on the Pittsburgh Sleep Quality Index (PSQI); 2.) Current presence of two or more risk categories on the Berlin Questionnaire for sleep apnea and overnight oximetry showing 10 desaturation events per hour or other results which are, in the judgment of the Investigator-Sponsor, suggestive of sleep apnea. 3.) A current or lifetime diagnosis of any psychiatric disorder with psychotic features, major depression, bipolar disorder, panic disorder, obsessive-compulsive disorder, posttraumatic

	stress disorder, generalized anxiety disorder, dysthymia, or agoraphobia without panic disorder, or current diagnosis of depressive disorder not otherwise specified, assessed using the Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) Axis I Disorders (SCID); 4.) A current diagnosis of alcohol or substance abuse or dependence or a history of alcohol or substance abuse or dependence within the past year, assessed using the SCID; 5.) Subjects who are pregnant, lactating, or planning to become pregnant or subjects who are not willing to use an acceptable form of birth control during the study; 6.) Lifetime history of brain injury (including concussions, mild traumatic brain injuries, or loss of consciousness for $\geq$ 10 minutes which resulted in the development of persistent symptoms lasting $\geq$ 1 month), stroke, brain hemorrhage, seizures (not including infantile febrile seizures), epilepsy, or brain infection caused by meningitis, encephalitis, or any other infectious agent. 7.) Systemic illness affecting central nervous system (CNS) function; 8.) Cardiovascular disease (to include but not limited to arrhythmias, valvular heart disease, congestive heart failure, history of myocardial infarction or family history of sudden cardiac death), hypertension, or hypercholesterolemia; 9.) Asthma or other reactive airway diseases; 10.) Hepatic impairment (Child-Pugh A, B, C); 11.) Any other chronic or unstable medical conditions; 12.) Current use of statins, ketoconazole, prescription or over-the-counter medications or herbal supplements containing psychoactive properties or stimulants in the judgment of the Investigator-Sponsor or Medical Monitor; 13.) Treatment with another investigational drug; 14.) Current daily use of any other medication unless specifically approved by the Investigator-Sponsor; 15.) Consumption of grapefruit (including grapefruit juice) or treatment with moderate or strong inhibitors of cytochrome P450 3A4 (CYP3A4) within one week prior to randomization; 16.) Treatment with drugs metabolized by CYP2D6 isoenzyme with a narrow therapeutic index within one week prior to randomization; 17.) Self-reported regular nicotine use within the past 30 days involving > 4 cigarettes per week or > 2 cigarettes per day; 18.) Self-reported consumption of alcohol within the past 30 days of >14 standard drinks per week or $\geq$ 5 standard drinks

	on any day (men), or > 7 standard drinks per week or ≥ 4 standard drinks on any day (women). 19.) Use of opioids, benzodiazepines, amphetamines, cocaine, cannabis, or any other illicit drugs within 30 days of screening by self report or a urine toxicology screen; 20.) Known liver disease or abnormal liver function tests assessed at the time of screening; 21.) Self-reported regular caffeine use in excess of 400 mg per day on average within six months of screening; 22.) Habitual long sleepers (> 9 hours) or short sleepers (< 5 hours); 23.) Shift work within one month prior to the screening visit or planned shift work during the study; 24.) Subjects who have traveled > 3 time zones within one week prior to the screening visit or any other visit; 25.) Known hypersensitivity or contraindication to any excipients of the drug formulation.
Outcome Measures:	<u>Primary Endpoints:</u> 1.) A comparison between groups on performance on the following neurocognitive measures: Rey Auditory Verbal Learning Test (RAVLT), Digit Span subtest of the Wechsler Adult Intelligence Scale IV (DS), Grooved Pegboard motor test, Paired-Associates subtest of the Wechsler Memory Scale (P-A), Stroop Color-Word Test (Stroop), Tower Test from Delis-Kaplan Executive Function System (D-KEFS Tower), Psychomotor Vigilance Test (PVT), and Conners' Continuous Performance Test II (CPT). 2.) A comparison between groups on latency to sleep onset measured by Maintenance of Wakefulness Tests (MWT) at 30 minutes and 150 minutes post-dose. 3.) A comparison between groups on low frequency EEG power during artifact free wake time as measured during MWTs. <u>Secondary Endpoints:</u> 1.) A comparison between groups on latency to sleep onset measured by MWTs at 270 and 390 minutes post-dose. 2.) A comparison between groups on Stanford Sleepiness Scale (SSS) scores. <u>Covariates:</u> 1.) Polysomnography (PSG) – Total Sleep Time on the night prior to the day of dosing. 2.) Actigraphy – Average sleep duration.
Statistical	It is hypothesized that subjects receiving zolpidem 10mg will show

Considerations:	greater impairment in neurocognitive performance compared to subjects receiving placebo, almorexant 100mg, or almorexant 200mg. This hypothesis will be tested by comparing groups on post-medication performance tests using pre-medication test scores as covariates. Where multiple administrations of a performance test are given either pre-or post-medication, mixed effects models will be used, with the group by time (i.e., pre- vs. post-medication) interaction effect serving as the test of the hypothesis. Where a test is administered only once pre- and post-medication, the statistical test will be a one-way ANCOVA comparing mean scores on the four groups, with the pre-medication test score serving as the covariate. Planned comparisons will be conducted to compare the zolpidem 10mg group with placebo, almorexant 100mg, and almorexant 200mg separately. P-value adjustments will be made for multiple endpoint variables within any given neurocognitive domain using a step-down non-parametric re-sampling-based procedure. Primary analyses will be intent-to-treat, including all subjects randomized regardless or dropout or missing data status. Missing data will be carefully characterized and multiply imputed if necessary.

1. INTRODUCTION

1.1 Background

In recent years, there has been increased focus on cognitive side effects associated with sleep-inducing medications that may contribute to unusual behavior during unexpected awakenings during the night. Concerns regarding these side effects have led to a Food and Drug Administration (FDA) class warning for all sleep-inducing medication. These concerns are particularly important to the military and other professions that have an occupational risk of poor sleep and being unexpectedly awakened with an expectation to perform without impairment.

Almorexant is a hypocretin/orexin antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Hypocretin/orexin is a neuropeptide system that stimulates arousal and is involved in sleep regulation. Disruption of the hypocretin/orexin system has been shown to result in the sleep disorder narcolepsy in both animals and humans, indicating that this system is part of the intricate sleep/wakefulness regulatory network. Hypocretin receptors are found in many brain regions, although receptor expression is weak in the cortex and high in brain regions associated with arousal state regulation, particularly the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems. Since the hypocretin peptides are excitatory throughout the brain, hypocretin antagonists work by blocking this excitation rather than producing a generalized inhibition. To the contrary, benzodiazepine receptor agonists (BzRAs) such as zolpidem affect gamma-aminobutyric acid ($GABA_A$) receptors which have widespread distribution in the central nervous system (CNS), particularly in the cerebral cortex. BzRAs therefore cause a general inhibition of neural activity (2).

1.1.1 Preclinical Background

Preclinical data demonstrate that almorexant produces a profile that is unique among currently marketed hypnotic medications. For example, preliminary study results in rats treated with one of three doses (10mg/kg, 30mg/kg, and 100mg/kg) of almorexant, zolpidem or placebo in the middle of the dark active period (six hours after lights offset) demonstrated that the 30mg/kg and 100mg/kg doses of almorexant and zolpidem increased non-rapid eye movement (NREM) sleep for several hours after dosing, whereas 10mg/kg of almorexant had a more transient effect. All three doses of almorexant increased rapid eye movement (REM) sleep while REM was suppressed by zolpidem. Consequently, the REM-NREM ratio was unchanged relative to vehicle in animals treated with almorexant, but zolpidem produced a decreased REM-NREM ratio which is characteristic of BzRAs. When cumulative effects were assessed over the entire six-hour post-treatment period, it was evident that almorexant produced a dose-dependent decrease of wake and a dose-dependent increase in both NREM and REM sleep. This profile of a proportional increase of REM and NREM sleep appears to be unique among currently marketed hypnotic medications (3).

Additionally, almorexant appears to have few side-effects on regulated physiological systems. Preliminary studies comparing the effects of varying doses of almorexant, zolpidem, and placebo on core body temperature in rats revealed that zolpidem-treated

animals experienced a significant and prolonged change in core body temperature post-treatment, but there was relatively little change in core body temperature associated with any dose of almorexant (3).

In studies involving somnolent rats treated with almorexant, the rats showed an immediate reversibility of the hypnotic effect with no impairment on motor performance tasks (3). If similar observations are confirmed in humans, there will be enormous implications for the management of disturbed sleep in both military and civilian populations.

1.1.2 Clinical Background

Because hypocretins are implicated in coordinating states of wakeful vigilance, there has been a rapid development of small molecule hypocretin 1 and hypocretin 2 antagonists for possible use in insomnia. At present, there are robust drug discovery programs for hypocretin1/hypocretin 2 antagonists sponsored by Actelion, Glaxo-Smith Kline, Merck, Banyu, Sanofi-Aventis, and Janssen. In 2007, Actelion presented results of a multi-site, double-blind placebo controlled trial in insomnia patients examining the effects of 50mg, 100mg, 200mg, and 400mg doses of almorexant at bedtime. The results showed significant improvement in sleep efficiency and reduced wake after sleep onset (WASO) at doses of 100mg and higher (4). There was no occurrence of cataplexy at any of the dosages used. Almorexant has an elimination half-life of 1.4 hours and effects on sleep electroencephalography (EEG) were absent after 6.5 hours (3).

Almorexant was well-tolerated in studies completed to date, including nineteen Phase I studies in healthy and hepatically impaired subjects, two dose-finding studies in adult and elderly patients with primary insomnia, and one Phase III study in primary insomnia. 519 healthy and hepatically impaired subjects were exposed to at least one dose of almorexant in Phase I studies. 633 subjects with primary insomnia have been exposed to at least one dose of almorexant in completed studies. Maximum exposure was up to 400mg daily for 1 day or up to 200mg for 16 days. 166 patients with primary insomnia received 200mg for at least 14 days, and 176 received 100mg for at least 14 days. The most frequently reported adverse events with almorexant were headache, fatigue, dizziness, and somnolence (40).

1.2 Rationale

At appropriate doses, all currently available FDA-approved prescription sleep-inducing agents induce restorative sleep. However, they also exert substantial performance-impairing effects at peak concentration in multiple domains of neurocognitive function. For example, multiple studies have shown impairment in driving within the six-hour window after ingesting zolpidem (6, 7). Other studies have documented impairment in balance and postural tone within two hours of taking zolpidem (8). Furthermore, there is solid evidence that at peak concentration, currently available sleep-inducing agents significantly impair the ability to consolidate new memories (9-12). This evidence therefore precludes the use of sleep-inducing agents under operational conditions in which individuals might be called upon to perform without impairment after taking the

agent, which is particularly relevant to populations involved in military combat. Further, there is an enormous accumulation of data linking disturbed sleep to a wide range of outcomes including daytime fatigue (13-15), impaired concentration and attention (16-19), increased risk for accidents and injuries (20, 21), worsened quality of life (22), increased aggression (23-26), and increased use of alcohol (27, 28). Several studies have also demonstrated that disturbed sleep is a potent risk factor for later onset development of major depression, panic disorder, alcohol, and substance abuse (27-30). Therefore, an effective treatment for sleep disturbances that can be safely utilized in deployed military personnel in combat operations without performance-impairing effects has the potential for improving the success of combat operations, inoculating soldiers against battlefield stress-related psychiatric illnesses, and preserving the psychological health of the soldiers throughout the full deployment lifecycle. The availability of such a treatment would also have a positive impact on the overall quality of life, physical, and psychological well being of the civilian population.

The study discussed in this protocol will involve a double-blind, placebo-controlled, randomized, parallel-groups study design and will involve a one-time oral administration of one of four dosing options to healthy volunteers: almorexant 100mg, almorexant 200mg, zolpidem 10mg, and placebo. These dosages have demonstrated favorable safety profiles in clinical trials (5). Subjects will be dosed at the average midpoint of the habitual wake period. Neurocognitive performance assessments will be administered at the time of peak plasma concentration. The study will establish whether almorexant is superior to zolpidem and placebo regarding neurocognitive performance at the estimated peak plasma concentration.

2. CLINICAL STUDY OBJECTIVES

2.1 Primary Objectives

Primary endpoints are listed below:

1.) A comparison between groups on performance on the following neurocognitive measures: Rey Auditory Verbal Learning Test (RAVLT), Digit Span subtest of the Wechsler Adult Intelligence Scale IV (DS), Grooved Pegboard motor test (GP), Paired-Associates subtest of the Wechsler Memory Scale (P-A), Stroop Color-Word Test (Stroop), Tower Test from Delis-Kaplan Executive Function System (D-KEFS Tower), Psychomotor Vigilance Test (PVT), and Conners' Continuous Performance Test II (CPT).
2.) A comparison between groups on latency to sleep onset measured by Maintenance of Wakefulness Tests (MWT) at 30 minutes and 150 minutes post-dose.
3.) A comparison between groups on low frequency EEG power during artifact free wake time as measured during MWTs.

2.2 Secondary Objectives

Secondary endpoints are listed below:

1.) A comparison between dosing groups on latency to sleep onset measured by MWTs at 270 and 390 minutes post-dose.
2.) A comparison between dosing groups on Stanford Sleepiness Scale (SSS) scores.

The following outcomes will be analyzed as covariates:
1.) Polysomnography (PSG) - Total Sleep Time on the night prior to the day of dosing.
2.) Actigraphy – Average sleep duration.

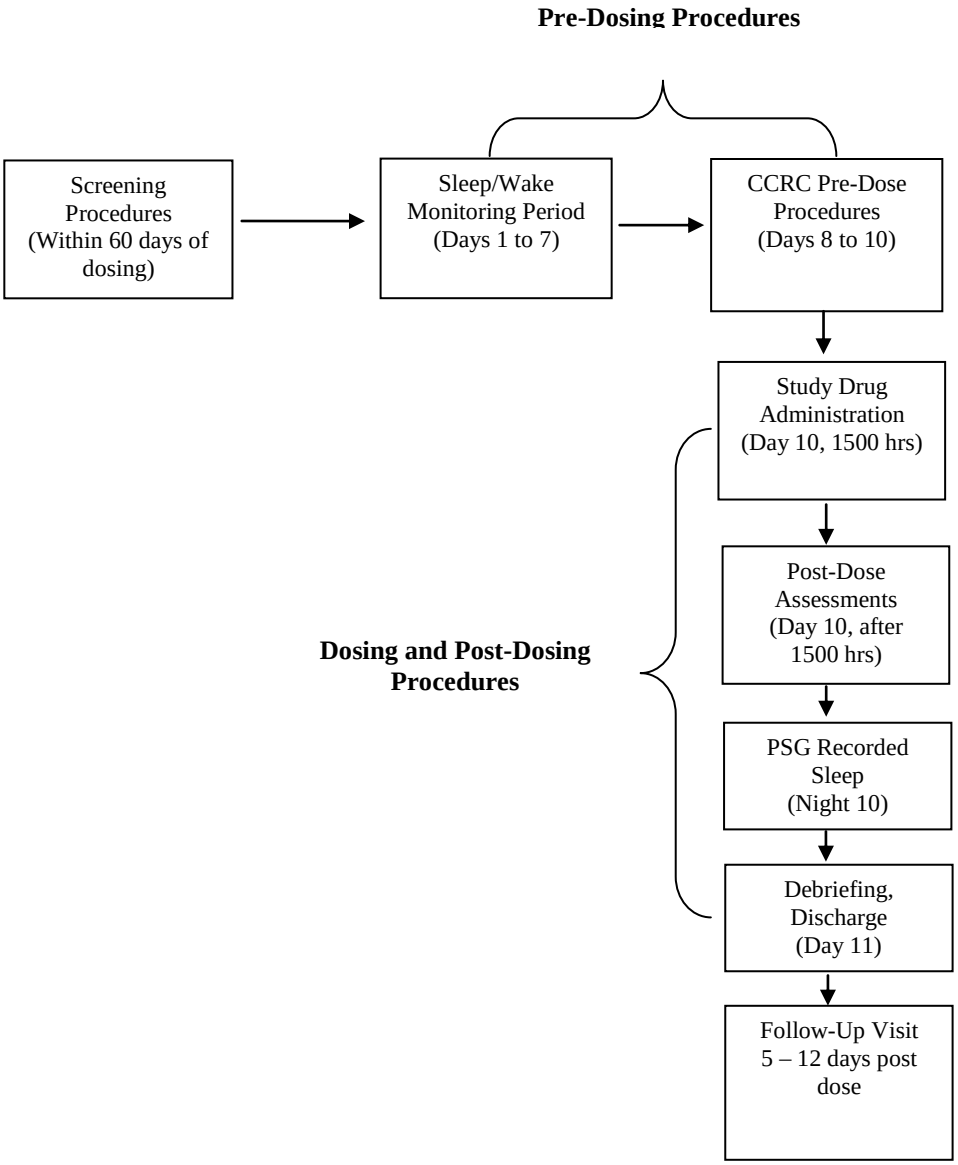
3. STUDY DESIGN

The study will take place at the San Francisco Department of Veterans Affairs Medical Center (SFDVAMC) and the University of California, San Francisco Clinical Translational and Sciences Institute inpatient Clinical Research Center (CCRC). The study will involve healthy volunteers who are considered normal sleepers per the Research Diagnostic Criteria for Normal Sleepers (1) as listed below:

1.) Subject has no complaints of sleep disturbance or daytime symptoms attributable to unsatisfactory sleep.
2.) Subject has a routine sleep/wake schedule characterized by regular bedtimes and rising times.
3.) There is no evidence of a sleep-disruptive medical or mental disorder.
4.) There is no evidence of sleep disruption due to a substance exposure, use, abuse, or withdrawal.
5.) There is no evidence of a primary sleep disorder.

Subjects will also be free of medical disorders and specified psychiatric disorders. After informed consent has been obtained and eligibility has been confirmed, subjects will be instrumented with wrist actigraphs to record their sleep/wake patterns for seven days; subjects will also be asked to complete a sleep diary during this one-week time period. Subjects will be admitted to the CCRC on the day after completion of the one-week sleep/wake monitoring period and two days prior to drug administration. Subjects' sleep will be monitored with PSG during each night at the CCRC, and sleep apnea will be screened for during the first night of PSG. Subjects will continue to maintain a sleep diary and wear an actigraph while at the CCRC. Subjects will be randomized in a double-blind fashion to one of four groups (almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo). An unblinded research pharmacist will provide study drug to a nurse at the CCRC for dispensing. The nurse and all other study personnel will remain blinded when study drug is dispensed to subjects. Following dosing, subjects will be accompanied by study personnel and instructed to remain awake. Neurocognitive, objective alertness, and subjective symptom assessments will be administered at regular intervals for several hours following dosing. Adverse events (AEs) will be assessed at the time of admission to the CCRC and on each day of the subject's stay in the CCRC. Subjects will be debriefed and discharged from the CCRC during the morning of the fourth day on the unit. They will be required to return to the SFDVAMC within 5 – 12 days of dosing for a safety lab test (liver function).

3.1 Study Design Schematic



4. SUBJECT SELECTION

Medically healthy men and women ages 19-39 (N = 216) will be recruited from newspaper advertisements, web based postings, websites, and flyers posted in various university and community sites. The age range is restricted to an upper limit of 39 years as a result of research showing a change in middle-aged individuals (defined as 40+ years of age) in terms of total sleep time and other sleep parameters that can affect performance outcomes independent of sleep deprivation and/or drug administration, which could therefore introduce a substantial source of error variance into the study (31). Interested potential subjects will be contacted by the study recruiter. If potential subjects agree, a 15 – 30 minute phone discussion will take place to determine whether they might be a match for the study. If the phone conversation indicates that the potential subjects may be a match for the study and they are still interested, they will be scheduled to meet with the study coordinator or another qualified study team member in person at the SFDVAMC for informed consent and further eligibility procedures.

4.1 Subject Inclusion Criteria

Subjects must meet all inclusion criteria in order to be eligible for the study:

1.) Male and female subjects between the ages of 19 and 39 determined to be physically healthy by physical exam and laboratory assessments;
2.) Habitual wake time between 0600 hr and 0900 hr maintained within the past month;
3.) Habitual bedtime between 2200 hr and 0100 hr maintained within the past month;
4.) Body Mass Index (BMI) >18 and < 28 kg/m^2;
5.) Ability to communicate well with the Investigator and to understand the study requirements.

4.2 Subject Exclusion Criteria

Any of the following criteria will exclude the subject from entering the study:

1.) Diagnosis of a sleep disorder within two years of screening or current sleep disturbance as suggested by a global score of > 5 on the Pittsburgh Sleep Quality Index (PSQI) (43);
2.) Current presence of two or more risk categories on the Berlin Questionnaire (42) for sleep apnea and overnight oximetry showing 10 desaturation events per hour or other results which are, in the judgment of the Investigator-Sponsor, suggestive of sleep apnea.
3.) A current or lifetime diagnosis of any psychiatric disorder with psychotic features, major depression, bipolar disorder, panic disorder, obsessive-compulsive disorder, posttraumatic stress disorder, generalized anxiety disorder, dysthymia, or agoraphobia without panic disorder, or current diagnosis of depressive disorder

not otherwise specified, assessed using the Structured Clinical Interview for DSM-IV TR Axis I Disorders (SCID) (41);

4.) A current diagnosis of alcohol or substance abuse or dependence or a history of alcohol or substance abuse or dependence within the past year, assessed using the SCID (41);
5.) Subjects who are pregnant, lactating, or planning to become pregnant or subjects who are not willing to use an acceptable form of birth control during the study;
6.) Lifetime history of brain injury (including concussions, mild traumatic brain injuries, or loss of consciousness for $\geq$ 10 minutes which resulted in the development of persistent symptoms lasting $\geq$ 1 month), stroke, brain hemorrhage, seizures (not including infantile febrile seizures), epilepsy, or brain infection caused by meningitis, encephalitis, or any other infectious agent.
7.) Systemic illness affecting central nervous system (CNS) function;
8.) Cardiovascular disease (to include but not limited to arrhythmias, valvular heart disease, congestive heart failure, myocardial infarction or family history of sudden cardiac death), hypertension, or hypercholesterolemia;
9.) Asthma or other reactive airway diseases;
10.) Hepatic impairment (Child-Pugh A, B, C);
11.) Any other chronic or unstable medical conditions;
12.) Current use of statins, ketoconazole, prescription or over-the-counter medications or herbal supplements containing psychoactive properties or stimulants in the judgment of the Investigator-Sponsor or Medical Monitor;
13.) Treatment with another investigational drug;
14.) Current daily use of any other medication unless specifically approved by the Investigator-Sponsor;
15.) Consumption of grapefruit (including grapefruit juice) or treatment with moderate or strong inhibitors of cytochrome P450 3A4 (CYP3A4) within one week prior to randomization;
16.) Treatment with drugs metabolized by CYP2D6 isoenzyme with a narrow therapeutic index within one week prior to randomization;
17.) Self-reported regular nicotine use within the past 30 days involving > 4 cigarettes per week or > 2 cigarettes per day;
18.) Self-reported consumption of alcohol within the past 30 days of >14 standard drinks per week or $\geq$ 5 standard drinks on any day (men), or > 7 standard drinks per week or $\geq$ 4 standard drinks on any day (women).
19.) Use of opioids, benzodiazepines, amphetamines, cocaine, cannabis, or any other illicit drugs within 30 days of screening by self report or a urine toxicology screen;
20.) Known liver disease or abnormal liver function tests assessed at the time of screening;
21.) Self-reported regular caffeine use in excess of 400 mg per day on average within six months of screening;
22.) Habitual long sleepers (> 9 hours) or short sleepers (< 5 hours);
23.) Shift work within one month prior to the screening visit or planned shift work during the study;

24.) Travel of > 3 time zones within one week prior to the screening visit or any other visit;
25.) Known hypersensitivity or contraindication to any excipients of the drug formulation.

5. STUDY DRUG HANDLING

5.1 Allocation to Dosing Groups

Subjects will be randomly assigned to one of four dosing groups in a 1:1:1:1 ratio: almorexant 100mg, almorexant 200mg, zolpidem 10mg, or placebo. Randomization will be stratified based on gender and caffeine use. Subjects will dose one time on Study Day 10 at 1500 hrs according to their assigned dosing group.

Almorexant (100mg and 200mg) is currently being investigated in a comprehensive Phase III program. Results indicate that almorexant was well-tolerated in the initial Phase III study. Further Phase III studies to evaluate long-term efficacy and safety are in preparation (4).

Zolpidem 10mg is an imidazopyridine class sedative hypnotic which received original United States market approval under the brand name Ambien® in 1992.

5.2 Breaking the Blind

The blind will be maintained through study completion except for cases of breaking the blind due to emergency medical necessity. In situations in which the CCRC nursing staff or other study personnel determines that it might be necessary to break the blind, he/she will be instructed to contact the Investigator-Sponsor or Medical Monitor. If approval is granted by the Investigator-Sponsor or Medical Monitor, the CCRC nurse will be authorized to contact the research pharmacist at the CCRC. The research pharmacist will maintain a master randomization list and he/she or an authorized designee will be available to break the blind if necessary.

5.3 Dosing Adherence/Study Compliance

Since only one dose will be administered to subjects by a nurse at the CCRC, deviations from the scheduled dosing regimen are not anticipated.

During the sleep-wake monitoring period which will take place throughout the week prior to admission to the CCRC, subjects will be required to maintain regular wake times between 0600 hr and 0900 hr and bedtimes between 2200 hr and 0100 hr. Additionally, subjects will be asked to avoid recreational drug use, naps, the consumption of grapefruit or grapefruit juice, alcohol, and/or nicotine. Subjects will also be asked to maintain stable caffeine use and to avoid crossing more than three time zones. Actigraphs will be utilized to monitor the subjects' sleep-wake patterns and will therefore serve as a check for compliance with the prescribed sleep regimen. Subjects will maintain daily sleep diaries

during the 10-day study period which will capture the following items: lights out and wake clock times, estimated sleep latency, wake time in minutes after sleep onset, rating of sleep quality on a scale of 1-100, caffeine use, and atypical events. Actigraphy and sleep diary data will be reviewed upon admission to the CCRC to determine compliance with the required sleep/wake schedule. An additional urine toxicology screening will be administered at the time of admission to the CCRC to rule out recent recreational drug use, and females will receive a urine pregnancy test at this time.

5.4 Drug Supplies

5.4.1 Formulation and Packaging

Actelion Pharmaceuticals Ltd. will provide almorexant 100 mg tablets, zolpidem 10 mg capsules, and matching placebo tablets and capsules. A double dummy design will be employed which will result in each subject receiving two tablets and one capsule. Study drug will be provided in bulk and will be shipped directly to the research pharmacy at the CCRC.

5.4.2 Preparing and Dispensing

The research pharmacist in the CCRC will maintain a copy of the randomization schedule and will receive the subject's randomization assignment at the time of hospital admission. The research pharmacist will dispense the assigned study drug to the nurse who will be administering the drug to the randomized subject.

5.4.3 Drug Administration

After obtaining the appropriate study drug from the research pharmacy, a CCRC nurse will administer the drug to the subject.

5.5 Drug Storage and Accountability

All drug products will be stored at the recommended temperature (room temperature at a maximum of 25^{o}C). Site personnel and study monitors will perform regular checks to document that the study drug is stored appropriately and is within the defined expiration period at all times. A drug accountability log will be completed by the research pharmacist when study drug is received and dispensed to subjects. Any unused drug will be destroyed at the conclusion of the study.

5.6 Concomitant Medications

Medication use will be assessed at screening. Concomitant medications will also be assessed when the subject arrives at the CCRC on Day 8, on each subsequent day in the CCRC (Days 9, 10, and 11), and at follow-up. All concomitant medications will be recorded in the source documents and transcribed onto the Case Report Forms (CRFs).

5.6.1 Disallowed Concomitant Medications and Dietary Restrictions

Use of statins, prescription or over-the-counter medications containing psychoactive properties or stimulants is exclusionary and is also prohibited during the study period. Subjects will be required to maintain stable caffeine consumption of 200 mg per day or less during the study. Alcohol, recreational drug, and nicotine use is prohibited during the 10 day study period. Consumption of grapefruit (including grapefruit juice) or treatment with moderate or strong inhibitors of cytochrome P450 3A4 (CYP3A4) within one week prior to randomization is prohibited.

6. STUDY PROCEDURES

6.1 Pre-Dosing Procedures

Screening

The study coordinator or another qualified, trained study team member will obtain informed consent from each potential subject prior to the initiation of eligibility procedures. During the informed consent meeting, the study will be explained and the subject's questions will be answered. Subjects will be allowed to take as much time as they need to make a decision and will be given the option of discussing their decision with their family, friends, or other healthcare providers.

- Physical Exam, Medical History, and Prior/Concomitant Medications Assessment (performed by a nurse practitioner at the SFVAMC CRC).
- Laboratory Analysis of Blood and Urine Samples: A urine sample and approximately 20ccs of blood will be collected for laboratory tests which will include a serum chemistry panel, liver function tests (including albumin), thyroid function tests, prothrombin time, complete blood count and differential, urine toxicology screen, and a urine pregnancy test (in women of childbearing potential). If lab values are out of range, subjects may be asked to repeat the blood draw for a retest to confirm their medical health.
- Structured Clinical Interview for Diagnostic and Statistical Manual of Mental Disorders (SCID [41]), performed by a trained mental health clinician
- Self-report Berlin Questionnaire (42) to determine likelihood of sleep disordered breathing. If subjects have two or more positive scoring categories, they will also be monitored with pulse oximetry.
- Self-report Pittsburgh Sleep Quality Index (PSQI [43])
- Review of Inclusion/Exclusion Criteria

All screening assessments will be performed at the SFDVAMC, including the collection of blood and urine samples and laboratory analysis. Dosing with study drug must take place within 60 days of when the screening assessments were administered. Screening assessments which were administered > 60 days prior to scheduled dosing will have to be repeated before subjects will be allowed to dose with study drug.

During the screening period the Vocabulary Subtest of the Wechsler Adult Intelligence Scale, Fourth Edition (WAIS-IV [48]) will be administered for the purpose of obtaining an IQ measure to ensure that all dosing groups are matched on intelligence. Vocabulary Subtest results will not be used to determine eligibility.

Sleep/Wake Monitoring (Days 1 to 7)

A seven-day sleep/wake baseline monitoring period will be scheduled for subjects who meet all inclusion and exclusion criteria. For female subjects, the baseline monitoring period will be scheduled such that Days 8 - 10 correspond to the follicular phase of the menstrual cycle. Prior to the start of the baseline week, a practice version of the PVT will be administered. Subjects will be asked to wear wrist actigraphs 24 hours per day on each day of the seven day monitoring period, and they will also be asked to abide by the following instructions:

- Adhere to a consistent wake schedule of 0600 hrs – 0900 hrs and a lights out schedule of 2200 hrs – 0100 hrs.
- Avoid nicotine and recreational drug use.
- Maintain stable caffeine consumption of ≤ 400 mg per day.
- Avoid alcohol use. (Although subjects will be encouraged to avoid alcohol entirely, acceptable use is ≤ 14 drinks per week or < 5 drinks on any day for men, and ≤ 7 drinks per week or <4 drinks on any day for women.)
- Avoid the consumption of grapefruit or grapefruit juice.
- Avoid travelling > 3 time zones.
- Avoid naps.
- Avoid starting new medications unless they become necessary in the opinion of a physician.
- Use an acceptable form of birth control.

Subjects will maintain daily sleep diaries during the sleep/wake monitoring period which will capture the following data points: lights out and wake clock times, estimated sleep latency, wake time in minutes after sleep onset, rating of sleep quality on a scale of 1-100, caffeine use, and atypical events.

Day 8 (CCRC Admission)

Subjects will enter the CCRC in the evening and a urine toxicology screen will be performed. A urine pregnancy test will be performed for female subjects of childbearing potential. Whether or not female subjects are in the follicular phase of their menstrual cycles will also be assessed at the time of admission. All subjects will be asked to report concomitant medications and AEs dating back to informed consent. Sleep diary data will be reviewed to determine compliance with the required sleep/wake regimen. Compliance with other study-related instructions will also be assessed at this time. While at the CCRC, subjects will receive a prescribed lights out time which will be consistent with the lights out regimen that was followed during the baseline week. All subjects will be prescribed a 0700hr wake time during their stay at the CCRC.

Subjects will continue to maintain a daily sleep diary and wear an actigraph during their stay at the CCRC. Additionally, during each night at the CCRC, subjects will have their sleep monitored with ambulatory PSG. Subjects will also be screened for obstructive sleep apnea which will involve thermistor measurements, pulse oximetry for detection of oxygen desaturation events, and two channels of respiratory inductive plethysmography to measure chest and abdominal movement during breathing. Subjects with an apnea/hypopnea index ≥ 10 will be excluded from the data analysis.

Day 9

Subjects will be awakened at 0700 hrs and will remain in the CCRC for monitoring. Caffeine consumption should remain consistent with what the subject consumed throughout the sleep/wake monitoring period, and caffeine will not be allowed after 1330 hrs. Naps will be prohibited. During the evening (prior to lights out), AEs and concomitant medications will be assessed. Subjects will have their sleep monitored with PSG.

Day 10 (Pre-Dose; 0700hrs – 1500hrs)

Subjects will be awakened at 0700 hrs. Caffeine consumption will remain consistent with what the subject consumed throughout the sleep/wake monitoring period, and caffeine will not be allowed after 1330 hrs. Beginning at 1000 hrs, subjects will be administered a series of baseline (pre-dose) neurocognitive assessments, objective alertness assessments, and subjective assessments. All assessments will be administered by qualified, trained, research technicians. Assessments to be administered are described below:

Stanford Sleepiness Scale: Subjects will be asked to rate themselves along a 7-point scale ranging from 1 (fully alert) to 7 (extremely sleepy). This scale will be administered just prior to each administration of the MWT. Administration time is less than 5 minutes.

Maintenance of Wakefulness Test: Subjects will be placed in a dimly lit room where they will sit comfortably and receive instruction to keep their eyes open and attempt to remain awake while being monitored via standard MWT EEG leads. If the subject falls asleep, he/she will be awakened after three epochs of sleep as determined by EEG trace. Administration time is 20 minutes.

Psychomotor Vigilance Test: Subjects will be required to press a button each time a target is presented. Administration time is 10 minutes.

Rey Auditory Verbal Learning Test – List 1: Each subject will be read a list of 15 words and asked to repeat back as many as they can remember. The task is repeated 4 more times. Subsequently, a new interference list is read and the subject is asked to repeat back items from that list. Then the subject is asked to recall items from the original list. Administration time is approximately 10 minutes.

Continuous Performance Test II: Subjects will be required to press the space bar or click the mouse button when any letter except for the target letter "X" appears. Administration time is 15 minutes.

Symptom Checklist: Subjects will be asked if they are experiencing specific symptoms commonly associated with hypnotics. If they endorse any of the symptoms on the checklist, they will be asked whether the symptoms are mild, moderate, or severe. Administration time is approximately 5 minutes.

Vital signs (sitting blood pressure and heart rate) will be obtained several times throughout Day 10. Staff will also query for AEs at these time points.

6.2 Study Dosing

Day 10 (Dosing and Post-Dose, 1500hrs - 2200hrs)

Subjects will dose at 1500 hrs. Shortly after dosing, a PVT administration will take place. MWTs (preceded by the SSS each time) will be administered at 1530 hrs, 1730 hrs, 1930 hrs, and 2130 hrs.

Based on the literature (3), it is estimated that almorexant will reach peak blood concentration between 1600 hrs and 1800 hrs. Around this timeframe, subjects will be administered the PVT, CPT, and SC, in addition to the MWT and SSS. The following neurocognitive assessments will also be administered during this timeframe:

Paired-Associates Learning Task: Subjects will be read 10 pairs of words. They will then be read, in a different order, the first word from each pair for which they are to recall the associated second word. The list will be presented and followed by recall two more times (with pairs in a different order each time). The first administration of the Paired-Associates Learning Task (given during the timeframe of 1600hrs – 1800hrs) will test immediate recall, during which errors are corrected. The test will be administered again several hours after the first administration using the same word list to assess delayed recall. Errors will not be corrected during the delayed recall trial.

Rey Auditory Verbal Learning Test – List 2: The RAVLT will be administered again during the 1600 – 1800hrs timeframe, but with a new list.

Grooved Pegboard Test: The test consists of 25 holes with randomly positioned slots. Pegs with a key along one side must be rotated to match the hole before they can be inserted and subjects must place the pegs in the holes as quickly as possible. Administration time is approximately 10 minutes.

Stroop Color-Word Test: Subjects will be given three sheets of paper, one at a time. The Word page consists of the words "red," "green," and "blue" printed randomly in rows in black ink. Subjects will be asked to read as many words as they can out loud in a 45 second time period. The Color page consists of 100 items, all written as "XXXX,"

printed in either green, red, or blue ink. Subjects will be asked to name as many colors as they can out loud in a 45 second time period. The Color-Word page consists of the words from the Word page printed in the colors from the Color page. The words and the colors they are printed in do not match one another. Subjects will be asked to name as many colors as they can in a 45 second time period. Total administration time is approximately 10 minutes.

Tower Test from Delis-Kaplan Executive Function System: Subjects will be asked to complete problem-solving tasks which will involve moving disks on pegs to match an arrangement shown to them in a picture. Administration time is approximately 20 minutes.

Digit Span: Subjects will be read a sequence of digits and asked to repeat the digits in the same sequence. For the second portion of the test, subjects will be read a sequence of digits and asked to repeat the digits in reverse order. For the third portion of the test, subjects will be read a sequence of digits and asked to repeat the digits in order from the lowest number to the highest. Administration time is approximately 6 minutes.

After the time window of 1600 hrs - 1800 hrs, subjects will receive additional administrations of the PVT, SC, and RAVLT (third list). Two more MWT administrations will also take place. The final assessment will begin at 2130 hrs.

Study personnel will remain with the subjects throughout testing and subjects will be kept awake until all assessments have been completed. Some of the neurocognitive tests will be audio recorded for quality control purposes.

Night 10 (Post-Dose)

AEs will be assessed prior to the prescribed lights out time. Subjects will engage in undisturbed, PSG recorded sleep.

Day 11 (Discharge)

Upon awakening at 0700 hrs, subjects will have all electrodes removed and will be debriefed prior to being discharged from the CCRC. AEs will be assessed prior to discharge.

Safety Follow-Up

Within 5 – 12 days of dosing with study drug, subjects will be required to have a blood draw performed for a liver function test. This procedure will be performed at the SFDVAMC. Approximately 5ccs of blood will be drawn and analyzed at the SFDVAMC laboratory. If lab values are out of range, the subject may be asked to repeate the blood draw for a retest. The occurrence of AEs and concomitant medications since the day of discharge will be assessed.

7. STUDY OUTCOMES AND SAFETY ASSESSMENTS

7.1 Study Outcome Assessment Measures

A description of the measures which will be utilized for the outcome analyses is provided below:

Psychomotor Vigilance Test: The PVT is a widely used instrument that measures sustained attention and reaction time (49). Extensive work with this measure has demonstrated that the PVT is not affected by practice effects and is a highly sensitive measure of the effects of disrupted circadian rhythms from shift work (17) and chronic sleep deprivation (18, 19). PVT-192® devices will be utilized for this study. The PVT has a random inter-stimulus interval of 2-10 seconds and can be collected over a 10 minute period. The main measure will be performance lapses (reaction time > 500 ms) per 10 minute period. Secondary measures will include total time of lapses, frequency of false responses, frequency of non-responses, durations of the 10% fastest and 10% slowest responses, and performance decrement across time on the task.

Stanford Sleepiness Scale: The SSS is a subjective measure of sleepiness in which subjects rate themselves along a 7-point scale ranging from 1 (fully alert) to 7 (extremely sleepy) (50). Subjective sleepiness ratings will be collected in order to verify the sedative effects of zolpidem and the two doses of almorexant.

Maintenance of Wakefulness Test: The MWT is widely used to demonstrate significant pre and post treatment differences in excessive sleepiness. Sleep onset is defined as the first occurrence of > 15 seconds of cumulative sleep in a 30 second epoch. Latency to the first 30 seconds of sleep will be scored online by the attending sleep technologist. The subject will be awakened within 90 seconds of falling asleep.

Rey Auditory Verbal Learning Test: The RAVLT is a word learning task and a measure of short-term auditory memory and learning, as well as delayed auditory memory (52, 53).

Grooved Pegboard Test: A measure of manipulative dexterity, this test requires complex visual-motor coordination (51).

Paired-Associates Learning Task: This associative learning sub-test of the Wechsler Memory Scale tests the ability to learn and recall pairs of words, some of which are related (e.g., north/south) and others which are unrelated (e.g., eagle/jury) (47). Immediate and delayed recall trials will be scored for the number of correctly recalled pairs.

Continuous Performance Test II: The CPT assesses attention and working memory as well as executive function (44). Specifically, the CPT measures response inhibition via commissions (an aspect of executive function) and sustained attention via omissions. There is evidence in the literature which suggests that continuous performance tasks are

sensitive to sleep-inducing agents (34). Scores will be based on response time and errors, inclusive of omissions and commissions.

Stroop Color-Word Test: The Stroop is a widely used putative measure of executive function that measures response inhibition (35). The Color-Word score will be computed, which measures the subject's ability to inhibit or override the tendency to produce the more automatic or dominant response (i.e., to name the color word rather than the color).

Tower Test from Delis-Kaplan Executive Function System: D-KEFS Tower is typically used for the assessment of executive function, specifically to detect deficits in planning, decision making, and problem solving (45). Literature provides evidence of a link between performance on towers tasks and sleep (32).

Digit Span: Digit Span is a subtest of the WAIS-IV which measures attention and working memory and has been found to be sensitive to sleep-inducing agents (36, 48).

The following measures will serve as covariates:

Actigraphy: The primary actigraph measures are habitual sleep onset and offset times and the range of variability around these data points. The wrist actigraph provides continuous activity data using a battery-operated wristwatch-sized microprocessor that senses motion with an accelerometer. Subjects can also indicate lights on, lights off, and other salient events by pressing an event marker on the actigraphs. The actigraphs will be initialized with the ActMe program (Ambulatory Monitoring, Inc.) using the PIM sampling mode in one-minute epochs for conventional actigraphic sleep-wake estimation.

Polysomnography: The primary PSG measure is total sleep time on the night prior to the day of dosing and neurocognitive testing. PSG recordings will be obtained with ambulatory PSG and the parameters recorded will follow current guidelines as defined in the AASM Manual for the Scoring of Sleep and Associated Events (37).

The Embla Titanium ambulatory recorders record up to 34 channels. The sampling frequency ranges from 256Hz to 512 Hz. High and low frequency filters will be added while scoring the data manually and in spectral analysis. 60Hz notch filters may be applied to remove electrical noise. Raw files will be kept with only anti-aliasing filters. Spectral analysis will organize sleep epochs by stage and time. Artifacts will be tagged for removal for spectral analysis.

7.2 Safety Assessment Measures

Symptom Checklist: This checklist captures common symptoms experienced by subjects taking hypnotic medications. Reports of symptoms will be collected in order to compare possible drug side effects.

AEs will be assessed on a regular basis throughout the study and at the follow-up visit.

A liver function test will be performed on all subjects within 5 – 12 days of dosing with study drug.

8. ADVERSE EVENT REPORTING

8.1 Adverse Event Definitions

An AE is defined as any untoward medical occurrence that takes place in a clinical study, regardless of the causal relationship of the event with the investigational drug or study treatment(s). Any event occurring after the clinical trial participant has signed the study informed consent documentation should be recorded and reported as an AE.

An AE can be any unfavorable and unintended sign, symptom, or disease temporally associated with the use of the investigational product, whether or not considered related to the investigational product. A new condition or the worsening of a pre-existing condition will be considered an AE.

An abnormal test finding will be classified as an AE if one or more of the following criteria are met: a.) the test finding is accompanied by clinical symptoms; b.) the test finding necessitates additional diagnostic evaluation(s) or medical/surgical intervention, including significant additional concomitant drug or other therapy; c.) the test finding leads to discontinuation of subject participation in the clinical study; d.) the test finding is considered an AE by the Investigator-Sponsor of the IND application.

For each AE, the date and time of onset, a description of the event, severity, seriousness, action taken, relationship to the study drug, outcome, and date of resolution will be recorded.

A **Serious Adverse Event (SAE)** is defined as an AE that results in any of the following:

- Death
- Life-threatening event – An event in which the subject is at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe.
- Requires hospitalization or prolongs existing inpatient hospitalization, not inclusive of a pre-planned elective hospital admission for treatment of a pre-existing condition that has not significantly worsened or a diagnostic procedure.
- Results in persistent or significant disability or incapacity.
- Results in congenital abnormality or birth defect.
- An important medical event occurs which requires medical intervention to prevent any of the above outcomes. Important medical events are those which may not be immediately life-threatening but may jeopardize the subject and may require intervention to prevent one of the serious outcomes listed above.

An **Unexpected Adverse Event** is defined as any AE in which the frequency, specificity, or severity is not consistent with the risk information described in the clinical protocol or elsewhere in the current IND application or Investigator's Brochure.

8.2 Recording Requirements

8.2.1 Eliciting Adverse Event Information

AEs will be assessed when subjects check into the CCRC and again during each evening at the CCRC. Additionally, subjects will complete a Symptom Checklist at various scheduled time points throughout the day of dosing and asked to report the occurrence of any other AEs.

8.2.2 Recording Requirements

All observed or volunteered adverse drug events (serious or non-serious) and abnormal test findings, regardless of treatment group or suspected causal relationship to the investigational drug or study treatment(s) will be recorded in the subjects' case histories. For all AEs, sufficient information will be pursued and/or obtained so as to permit a.) an adequate determination of the outcome of the event; and b.) an assessment of the causal relationship between the AE and the study drug.

AEs or abnormal test findings felt to be associated with the investigational drug or study treatment(s) will be followed until the event (or its sequelae) or the abnormal test finding resolves or stabilizes at a level acceptable to the Investigator-Sponsor.

8.3 Reporting of Adverse Events

8.3.1 Reporting of Adverse Events to the FDA

Written IND Safety Reports

The Investigator-Sponsor will submit a written IND Safety Report to the responsible new drug review division of the FDA for any observed or volunteered AE that is determined to be a.) associated with the investigational drug or study treatment(s); b.) serious; and c.) unexpected. Each IND Safety Report will be prominently labeled, "IND Safety Report."

Written IND Safety Reports will be submitted to the FDA as soon as possible and within 15 calendar days following the Investigator-Sponsor's receipt of the respective AE information. For each written IND Safety Report, the Investigator-Sponsor will identify all previously submitted IND Safety Reports that addressed a similar AE experience and will provide an analysis of the significance of newly reported AE in light of the previous, similar report(s).

Follow-up information to an IND Safety Report will be submitted to the applicable review division of the FDA as soon as the relevant information is available. If the results of the Investigator-Sponsor's follow-up investigation show that an AE that was initially determined to not require a written IND Safety Report does, in fact, meet the

requirements for reporting; the Investigator-Sponsor will submit a written IND Safety Report as soon as possible and within 15 calendar days after the determination was made.

In accordance with FDA requirements, annual safety reports will be submitted to the FDA.

Telephoned IND Safety Reports

In addition to the subsequent submission of a written IND Safety Report (i.e., completed FDA Form 3500A), the Investigator-Sponsor will notify the responsible review division of the FDA by telephone or facsimile transmission of any observed or volunteered AE that is a.) associated with the use of the investigational drug or study treatment(s); b.) fatal or life-threatening; and c.) unexpected.

The telephone or facsimile transmission of applicable IND Safety Reports will be made as soon as possible but in no event later than 7 calendar days after the Investigator-Sponsor's initial receipt of the respective human AE information.

8.3.2 Reporting Adverse Events to the Responsible IRBs

In accordance with applicable IRB policies of the Veterans Affairs Medical Center Research and Development Committee, University of California, San Francisco Committee on Human Research, and the U.S. Army Medical Research and Materiel Command Human Research Protection Office (USAMRMC HRPO), the Investigator-Sponsor will report, to the IRBs, any observed or volunteered AE that is determined to be associated with the investigational drug or study treatment(s), serious, and unexpected. AE reports will be submitted to the IRBs in accordance with the respective IRB procedures.

Applicable AEs will be reported to the IRBs as soon as possible and, in no event, later than 10 calendar days following the Investigator-Sponsor's receipt of the respective information. Follow-up information to reported AEs will be submitted to the IRB as soon as the relevant information is available. If the results of the Investigator-Sponsor's follow-up investigation show that an AE that was initially determined to not require reporting to the IRB does, in fact, meet the requirements for reporting, the Investigator-Sponsor will report the AE to the IRB as soon as possible, but in no event later than 10 calendar days after the determination was made.

In accordance with the USAMRMC HRPO requirements, unanticipated problems involving risk to volunteers or others, serious adverse events related to participation in the study and all subject deaths related to participation in the study should be promptly reported by phone (310-619-2165), by e-mail (hsrrb@amedd.army.mil), or by facsimile (301-619-7803) to the USAMRMC HRPO. A complete written report should follow the initial notification. In addition to the methods above, the complete report can be sent to the USAMRMC, ATTN: MCMR-ZB-P, 504 Scott Street, Fort Detrick, Maryland, 21702-5012.

The Medical Monitor is required to review all unanticipated problems involving risk to subjects or others, serious adverse events and all subject deaths associated with the protocol and provide an unbiased written report of the event to the USAMRMC HRPO. At a minimum, the Medical Monitor should comment on the outcomes of the event or problem and in the case of a serious adverse event or death, comment on the relationship to participation in the study. The Medical Monitor should also indicate whether he/she concurs with the details of the report provided by the Investigator-Sponsor. Reports for events determined by either the Investigator-Sponsor or Medical Monitor to be possibly or definitely related to participation and reports of events resulting in death will be promptly forwarded to the HRPO.

8.3.3 Reporting of Adverse Events to Actelion Pharmaceuticals

Copies of all periodic safety reports (including draft versions for review) to be submitted to the FDA will be provided to Actelion at least 10 days prior to their submission to the FDA. Copies of any MedWatch forms submitted to the FDA will be provided to Actelion immediately upon submission to the FDA.

All serious adverse events, regardless of causality and expectedness, will be reported to Actelion within 24 hours of the Investigator-Sponsor's knowledge of the event.

8.3.4 Withdrawal of Subjects Due to Adverse Events

Withdrawal of subjects due to an AE can take place at any time during the study at the discretion of the Investigator-Sponsor. Subjects may also choose to discontinue participation at any time.

9. STATISTICAL METHODS/DATA ANALYSIS

9.1 Study Endpoints

9.1.1 Analysis of Primary Endpoints

It is hypothesized that subjects receiving zolpidem 10mg will show greater impairment in neurocognitive performance and objective measures of sleepiness compared to subjects receiving placebo, almorexant 100mg, or almorexant 200mg. This hypothesis will be tested by comparing groups on post-medication performance tests using pre-medication test scores as covariates. When multiple administrations of a performance test are given either pre-or post-medication, mixed effects models will be used, with the group by time (pre- or post-medication) interaction effect serving as the test of the hypothesis. When a test is administered only once pre- and post-medication, the statistical test will be a one-way ANCOVA comparing mean scores on the four groups, with the pre-medication test score serving as the covariate. Covariates in all models will include total sleep time measured by PSG on the night before testing and average sleep duration measured by actigraphy. Planned comparisons will be conducted to compare the zolpidem 10mg group

with placebo, almorexant 100mg, and almorexant 200mg separately. Post-hoc comparisons will be made to compare placebo vs. almorexant 100mg, placebo vs. almorexant 200mg, and almorexant 100mg vs. almorexant 200mg. For post-hoc comparisons, p-value adjustments will be made using a re-sampling procedure as implemented in the SAS "simulate" adjustment option.

Two-tailed significance tests will be conducted at the p = .05 level. P-value adjustments will be made for multiple endpoint variables within each domain of neurocognitive functioning (verbal memory, attention/working memory, motor skills, executive function, and psychomotor vigilance) and objective sleepiness (sleep onset latency and low frequency EEG power in the MWT). The p-value adjustments will be made using a step-down, re-sampling based procedure (38, 39) which takes into account the correlational structure among the multiple variables. Primary analyses will be intent-to-treat analyses based on all participants randomized, regardless of dropout or missing data status. Dropout rate will itself be analyzed as a secondary outcome variable. Missing data will be carefully characterized, and multiple imputation will be used where necessary. The exact form of each mixed model, for example the correlational structure of repeated measures and whether heterogeneous group variances are included, will be made on the basis of best fit according to the Bayesian Information Criterion (BIC) before any hypothesis testing is conducted. Assumptions of the models (e.g., normal distributions of errors and absence of outliers) will be assessed, and any necessary remedies, such as data transformation or the use of robust standard errors, will be implemented before hypothesis tests are conducted.

Any deviations from the statistical plan will be described in the study manuscript.

9.1.2 Analysis of Secondary Endpoints

Secondary endpoints include sleep latency on the MWT measured beyond the presumed drug activity period at 270 and 390 minutes post-dose (i.e., the "hangover effect"), and subjective sleepiness measured by the Stanford Sleepiness Scale. Secondary analyses will be conducted in a parallel fashion to the primary analyses, but with re-sampling based multiple comparison procedures for all significance tests.

9.2 Sample Size Determination

Enrollment is estimated to include up to 216 subjects to obtain 200 evaluable subjects. An equal number of subjects (up to 54) will be randomly assigned to each dosing group (almorexant 100 mg, almorexant 200 mg, zolpidem 10 mg, placebo). Randomization will be stratified on the basis of gender and caffeine use. With a power of 0.80 and an alpha of 0.05, the planned sample size will allow for the detection of effect sizes (Cohens' f) of approximately 0.29. It is estimated that the effect of zolpidem 10 mg versus placebo on the cognitive performance measures will range from f = 0.34 to f = 0.80, based on prior findings. Given the hypothesis that both doses of almorexant will be associated with significantly less impairment than zolpidem 10mg, it is possible that a range of effect sizes might be found with almorexant. If almorexant is absolutely no different than

placebo, the study will be slightly overpowered to demonstrate its superiority over zolpidem. However, if almorexant has a more subtle impairment effect on cognition, intermediate between that seen with zolpidem 10 mg and placebo, it might become necessary to be able to detect somewhat smaller effects. According to guidelines suggested by Cohen (33), an effect size of f = .14 is considered "small" and f = .39 is considered "medium." Thus, the proposed study is well powered to test its main hypotheses.

9.3 Definition of Analysis Populations

Primary analyses will be intent-to-treat analyses based on all participants randomized, regardless of dropout or missing data status. If there are a substantial number of participant dropouts, separate analyses on completers only will be conducted as a sensitivity analysis, but hypothesis tests will be based on the intent-to-treat sample. No subgroup analyses are planned.

9.4 Safety Analysis

Dosing groups will be compared on each symptom included as part of the Symptom Checklist using Fisher's exact tests or Chi-Square approximations, depending on the frequency of each symptom. No p-value adjustments will be made.

10. QUALITY CONTROL (QC) AND QUALITY ASSURANCE

The study will be carried out according to requirements of the FDA and all other applicable agencies in addition to ICH accepted standards of GCP. All study-specific procedures will be performed according to approved written Standard Operating Procedures. Study monitors will be responsible for ensuring adherence to FDA and ICH guidelines. Study Monitors for this study will be provided by an external contract monitoring group. Regular monitoring of study data and files at the clinical study sites will be performed as defined in the study-specific monitoring plan. Additionally, an authorized representative from the Investigator-Sponsor study team will perform an annual review of study files and training files to ensure adherence to GCP guidelines and study-specific standard operating procedures. Data collected during the study will be subjected to a thorough quality control review by the lead data managers prior to the statistical analysis. Specific requirements related to the data management QC of the study data will be detailed in the Data Management Plan. AE data will be reviewed on an ongoing basis with the Investigator-Sponsor.

11. DATA HANDLING, RECORD KEEPING, AND CONFIDENTIALITY

11.1 Data Recording/Case Report Forms (CRFs)

A CRF will be completed for each subject enrolled into the clinical study. The Investigator-Sponsor will review each completed CRF book and will complete the Investigator Statement. Completion of the Investigator Statement CRF confirms the

Investigator-Sponsor's responsibility for ensuring that all data and corrections on the CRF are complete, accurate, and authentic.

Source documents will consist of laboratory and medical history records, screening instruments, actigraphy data, sleep diaries, PSG data, neurocognitive assessments, and subjective symptom measures including the Symptom Checklist, the Stanford Sleepiness Scale, and AE and concomitant medication disclosures. All necessary information from the source documents will be recorded on the CRFs. Where appropriate, certain data files will be merged with the study database electronically. Data recorded on the CRFs will be identical to the data recorded on the source documents. Queries will be issued to address all discrepancies noted within the study data. Any changes made to the study data as the result of a resolved query will be documented in the audit trail within the study database. Specific procedures related to the handling of blank, discrepant, or otherwise spurious data will be detailed in the Data Management Plan. When all data have been entered, validated and queries resolved, the database will be locked.

11.2 Record Maintenance and Retention

The Investigator-Sponsor will maintain records in accordance with GCP guidelines and all applicable regulations and policies, to include:

- FDA correspondence related to the IND and clinical protocol, including copies of submitted Safety Reports and Annual Reports
- IRB correspondence (including approval notifications) related to the clinical protocol, including copies of AE reports and annual or interim reports
- Current and past versions of the IRB-approved clinical protocol and corresponding IRB-approved consent form(s) and, if applicable, subject recruitment advertisements
- Signed FDA Form 1572 Statements of Investigator
- Financial disclosure information
- Curriculum vitae for the Investigator-Sponsor and all clinical protocol sub-investigators and study personnel
- Certificates of required training for Investigator-Sponsor, all sub-investigators, and other relevant study team members
- Listing of printed names/signatures of Investigator-Sponsor and listed sub-investigators
- Normal values for laboratory ranges
- Laboratory certification information
- Instructions for on-site preparation and handling of the investigational drug, other study treatments, and study materials
- Standard procedures for decoding and breaking the study blind
- Master randomization list
- Signed informed consent forms
- Completed Case Report Forms, signed and dated by the Investigator-Sponsor
- Source Documents

- Monitoring visit reports
- Copies of Investigator-Sponsor correspondence to sub-investigators, including notifications of safety information
- Subject screening and enrollment logs (a listing of all volunteers who signed informed consent)
- Subject identification code list
- Investigational drug dispensing and accountability records, including documentation of drug disposal
- Final clinical study report

The Investigator-Sponsor will retain the specified records and reports for a minimum of two years after the marketing application is approved for the investigational drug. If a marketing application is not submitted or approved for the investigational drug, records will be retained until 2 years after investigations under the IND have been discontinued and the FDA so notified.

11.3 Confidentiality

Participation in research will involve a loss of privacy, but information about subjects will be handled as confidentially as possible. Medical records will be created at UCSF and SFVAMC because of subjects' participation in this study. Information related to informed consent and screening test results will be included in the medical records, as well as information pertaining to vital signs, adverse events, and concomitant medications assessed during the hospital portion of the study. Therefore, other doctors may become aware of the individual's study participation. Hospital regulations require that all health care providers treat information in medical records confidentially. At the time of consent, subjects will be asked to sign forms to authorize the release of their personal health information for research purposes.

If it is suspected that the subject is in danger of harming him/herself or someone else, or if child abuse or neglect or elder abuse has occurred, appropriate authorities will be notified as required by law. It is also possible that subjects' research records could be subpoenaed by a court.

If information from this study is published or presented at scientific meetings, subjects' names and other personal information will not be used.

All study data will all be coded with a code number unique to the study. Only study personnel, with the permission of the Investigator-Sponsor, will have access to the key with the name and ID codes. The subject identification code list will be stored electronically in a password-protected, restricted access folder on a secured study server in order to maintain confidentiality. The only individuals receiving access to the code list will be the team member responsible for maintaining the list and a back-up.

The clinical interviews performed at screening will be audio recorded and will be used only by research personnel in order to calibrate the clinicians' ratings on the standardized

interview format. The neurocognitive assessments performed on Day 10 will also be recorded for QC and calibration purposes. All recordings will be labeled with a unique code number and retained in a secure location (digital recordings will be encryped, passcode protected, and stored and accessed via the secure VA server). Recordings will be retained until the conclusion of the study; at that point, they will be erased. Subjects will be informed that their screening clinical interviews will be audio recorded for the purpose of allowing the research team to ensure consistency across all clinical interviews. They will be informed that the recordings will be maintained under secure conditions at all times and identified only by the unique Subject ID number. Subjects will also be informed that the recordings will be deleted after the conclusion of the study.

The Maintenance of Wakefulness Tests performed on Day 10 will be video recorded and will be used only by research personnel for the purpose of confirming subjects' ability to remain awake during the testing process. The recordings will be labeled with a unique code number and retained in a secure location (digital recordings will be encryped, passcode protected, and stored and accessed via the secure VA server). Recordings will be retained until the conclusion of the study; at that point, they will be erased. Subjects will be informed that their Maintenance of Wakefulness Tests on Day 10 will be video recorded for the purpose of allowing the research team to confirm their ability to remain awake during testing. They will be informed that the recordings will be maintained under secure conditions at all times and identified only by the unique Subject ID number. Subjects will also be informed that the recordings will be deleted after the conclusion of the study.

Organizations that may look at and/or copy subjects' medical records for research, quality assurance, and data analysis include representatives from the following:

- UCSF CHR
- FDA
- USAMRMC
- Actelion Pharmaceuticals, Ltd.

12. ETHICS

12.1 Institutional Review Board (IRB) approval

Prior to initiating the study, the Investigator-Sponsor will obtain approval in writing from all required IRBs. Specifically, approval must be obtained from the UCSF Committee on Human Research, the Veterans Affairs Research and Development Committee, and the U.S. Army Medical Research and Materiel Command Office of Research Protections Human Research Protection Office.

Any amendments to the protocol or changes to the informed consent document must be approved by all IRBs prior to the implementation of those changes. The only circumstance in which a modification to the current IRB-approved clinical protocol/consent form(s) may be initiated in the absence of prospective IRB approval is

to eliminate an apparent immediate hazard to the research subject(s). In such circumstances, the Investigator-Sponsor will promptly notify the IRBs of the modification.

The IRBs will be promptly notified of any deviation to the protocol that may have an effect on the safety of the subjects and the integrity of the study. This notification will occur as soon as the deviation is identified. All deviations will also be reported in the continuing review report and final study report.

A copy of the approved continuing review report and the local IRB approval notification will be submitted to the USAMRMC ORP HRPO as soon as these documents become available. A copy of the approved final study report and local IRB approval notification will be submitted to the USAMRMC ORP HRPO as soon as these documents become available.

In the event that the IRB requires, as a condition of approval, substantial changes to a clinical protocol submitted under an FDA-accepted IND application, or in the event of an Investigator-Sponsor's decision to modify the previously accepted clinical protocol, the Investigator-Sponsor will submit a protocol amendment (prior to the implementation of the changes) to the IND describing any change to the protocol that would significantly affect the safety of subjects, the scope of the investigation, or the scientific quality of the study.

Records of IRB approval and other related correspondence will be maintained in the regulatory files for the study and will be subject to periodic audits and reviews by study monitors. Periodic status reports will be submitted to the IRB as required, and AEs/serious AEs will be reported to each IRB per their specific reporting requirements.

12.2 Ethical and Scientific Conduct of the Clinical Study

The clinical study will be conducted in accordance with the current IRB-approved clinical protocol, ICH Guidelines on GCP, and relevant policies, requirements, and regulations of the FDA, UCSF CHR, the VA R&D Committee, the USAMRMC ORP HRPO, and all other applicable state and federal agencies. All procedures described in this protocol will be performed according to approved written SOPs unless otherwise stated.

12.3 Subject Informed Consent

The Investigator-Sponsor will make certain that an appropriate informed consent process is in place to ensure that potential research subjects are fully informed about the nature and objectives of the clinical study, the potential risks and benefits of study participation, and their rights as research subjects. The Investigator-Sponsor, or a staff member designated by the Investigator-Sponsor, will obtain the written, signed informed consent of each subject prior to performing any study-specific procedures. The date and time that the subject signs the informed consent form and a narrative of the issues discussed during

the informed consent process will be documented in the subject's case history. The Investigator-Sponsor will retain the original copy of the signed informed consent form and a copy will be provided to the subject.

The Investigator-Sponsor will make certain that appropriate processes and procedures are in place to ensure that ongoing questions and concerns of enrolled subjects are adequately addressed and that the subjects are informed of any new information that may affect their decision to continue participation in the clinical study. In the event of substantial changes to the clinical study or the risk-to-benefit ratio of study participation, the Investigator-Sponsor will obtain the informed consent of enrolled subjects for continued participation in the clinical study

13. EARLY DISCONTINUATION CRITERIA

A subject may withdraw or be withdrawn from the study for the following reasons:

1.) Subject withdrew consent
2.) Investigator judgment
3.) Protocol violation(s)
4.) Non-compliance
5.) Adverse Event
6.) Pregnancy
7.) Other

If subjects withdraw consent prior to admission to the CCRC, they will be asked to return to the SFDVAMC for an early discontinuation visit which will entail an assessment of AEs and concomitant medications, a debriefing, and the return of study-related equipment.

If it becomes necessary to stop parts or all of the clinical study for the safety of the subjects, Actelion, the IRBs, and the FDA will be notified promptly of the discontinuation of the entire clinical study. Respective protocol modifications will be submitted prospectively to the IRB and to the FDA for discontinuation of parts of the clinical study. All sub-investigators will be notified of any necessary discontinuations.

Subjects participating in the study at the time of the discontinuation of a portion or all of the study will be promptly notified and advised of the impact of the discontinuation upon their study schedules. If a portion of the study is discontinued, subjects will be provided with revised informed consent documentation which will need to be signed prior to their continued enrollment in the study.

14. RISKS AND BENEFITS

Study-related risks and associated measures to minimize the risks are listed below:

Study Drug Related Side Effects

Some subjects might experience side effects associated with the study drugs. The list of possible side effects presented below is based on side effects that have been observed in clinical trials involving Almorexant and Zolpidem. Participants in these clinical studies took many different dosages of these drugs ranging from 1mg to 1000mg. Subjects will be told to discuss any side effects with study personnel as they occur. The nursing staff at the CCRC and study personnel will also closely monitor subjects on the day of dosing with study drug. All subjects will have a liver function test performed within 5 – 14 days of dosing with study drug.

Risks and side effects related to taking Almorexant include those which are:

Likely (occurring in greater than 20% of people)

- Drowsiness

Less Likely (occurring in less than or equal to 20% of people)

- Fatigue
- Headache
- Dizziness
- Nausea
- Liver Enzyme Elevations (mainly with administration for longer than two weeks of daily almorexant 100mg and 200mg)

Rare but Serious

- Heart rate abnormality (less than 1%)
- Convulsions (less than 1%)

Risks and side effects related to taking Zolpidem include those which are:

Less Likely (occurring in less than or equal to 20% of people)

- Dizziness
- Drowsiness
- Headache
- Diarrhea
- Fatigue

Rare but Serious

- Heart rate abnormality (less than 1%)
- Severe allergic reaction (less than 1%)

Blood Drawing (Venipuncture)

The risks of drawing blood include temporary discomfort from the needle stick, bruising, and rarely, infection. The amount of blood collected to determine eligibility is approximately 20 ccs or 4 teaspoons. Only a qualified phlebotomist will draw blood following standard SFVAMC lab procedures.

Clinical Interview & Questionnaires

The interview and questionnaires may be distressing to some participants. Subjects will be told that they are free to decline to answer any questions or to stop the interviews at any time.The interviewer will be available to immediately assist with any problems that arise in the interview and will make a referral if required.

Audio Recording – Clinical Interview and Neurocognitive Tests
The clinical interviews and some of the neurocognitive tests will be audio taped. The audio taping may make subjects somewhat more uncomfortable than they would be without the taping. Research personnel will use the recordings in order to ensure that study staff are administering and scoring the tests correctly and in the same way. The audio recordings will be maintained under secured conditions (i.e., the recordings will be encrypted, protected with a pass code, and stored and accessed via a secure server), identified only by a unique ID number, and retained until the conclusion of the study, at which point they will be erased/deleted.

Actigraphy
There is no risk of injury from wearing the actigraph. Subjects might find it annoying to have to wear the actigraph 24 hours per day during the 10 day study. Subjects will be told that they can discuss any difficulties with this procedure with study personnel at any time. Subjects will also be able to decline to participate in this procedure at any time.

Polysomnography
There is no risk of injury from any of the recording devices, but subjects might experience slight discomfort from the attached electrodes and tape. High quality hypoallergenic materials will be used to minimize this risk.

Video Recording – Maintenance of Wakefulness
The Maintenance of Wakefulness Tests that will be conducted on Study Day 10 will be videotaped. The video recording may make subjects somewhat more uncomfortable than they would be without the taping. These recordings will only be reviewed by research staff and our consultants for the purpose of confirming subjects' ability to remain awake during the testing. The recordings will be identified by a unique ID number and will be stored under secure conditions (i.e., they will be encrypted, protected with a pass code and stored on a secure server). The recordings will be retained until the conclusion of the study, at which point they will be destroyed.

Maintenance of Wakefulness Tests
There is no risk of injury from taking this test, but subjects might find it annoying or difficult to remain awake while sitting quietly in a comfortable position. Subjects might also become bored while sitting still for the 20 minute duration of the test. Subjects will be able to stop the procedure at any time if they become uncomfortable.

Neurocognitive Assessment Battery
There is no risk of injury from completing the neurocognitive assessment battery, but subjects might become bored, frustrated, or find it difficult to concentrate as you take these tests throughout the day of testing. Subjects will be able to stop the procedures at any time if they become uncomfortable.

Sleepiness

There is a 3 out of 4 chance that subjects will take a sleep aid on Study Day 10 while at the hospital. Therefore, subjects might become sleepy during the study testing procedures, and the study staff will require subjects to remain awake. This might be difficult or frustrating for subjects.

Reproductive Risks
Subjects should not become pregnant or father a baby while participating in this study because the potential effects of the study drugs on an unborn baby are not known at this time. Women should not breastfeed a baby while on this study. Study staff will educate subjects regarding the importance of using appropriate birth control throughout the study.

Unknown Risks
The experimental drugs used in this study may have side effects or discomforts that no one knows about yet. Subjects will be told to discuss any side effects with study personnel as they occur. The nursing staff at the CCRC and study personnel will also closely monitor subjects on the day of dosing with study drug.Subjects will not experience any direct benefits by participating in the study. However, the study is contributing to medical knowledge related to the cognitive effects of sleep aids. Results could have implications for personnel of the military and/or other professions who have an occupational risk of poor sleep.

15. STUDY PERSONNEL

15.1 Investigator-Sponsor

The Investigator-Sponsor will assume overall scientific and administrative leadership for the study. He will be responsible for supervising the study team with regards to the recruitment, diagnostic assessment, and enrollment of subjects and the coordination of all study procedures.

The Investigator-Sponsor will have overall responsibility for the standardization of data collection, data quality control, data analysis, and interpretation. He will have overall responsibility for subject safety, rights, and welfare. He will be an active participant in the preparation of abstracts and manuscripts and will assure the dissemination of study findings in the professional and scientific communities.

15.2 Medical Monitor

The Medical Monitor may be asked to discuss research progress with the Investigator-Sponsor, consult on individual cases, or evaluate adverse event reports for the safety and protection of the subjects. The Medical Monitor shall promptly report discrepancies or problems to the IRB and the HRPO, and he will have the authority to stop a research study in progress, remove individual subjects from a study, and take whatever steps are necessary to protect the safety and well-being of research volunteers until the IRB can assess the Medical Monitor's report. At a minimum, the Medical Monitor will provide a

written opinion regarding the relationship and outcome of any unanticipated problems related to participation, serious adverse events, and subject deaths.

15.3 Co-Investigators

The Co-Investigators assigned to this study will assist the research team in data collection, data analysis, quality control of study data, data interpretation, and the preparation of reports. They will provide consultation and oversight to the mental health clinicians and will assist with the determination of eligibility.

15.4 Study Coordinator

The study coordinator will be responsible for the day-to-day activities of the study, including but not limited to the following: obtaining informed consent, subject scheduling, eligibility determination, ensuring the completion of safety reports in a timely manner, case report form completion, ensuring that study team members are properly trained on study procedures, providing oversight to the external study monitors, and providing oversight for data completion, cleaning, analysis, and interpretation. The study coordinator will consult with the project director as necessary for high-level study management and budget oversight.

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Appendix 2: Animal Studies Progress Report

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Award Number:

USAMRAA Grant W81XWH-09-2-0081

TITLE:

EFFECT OF A HYPOCRETIN/OREXIN ANTAGONIST ON NEUROCOGNITIVE PERFORMANCE

PRINCIPAL INVESTIGATOR:

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13. SUPPLEMENTARY NOTES

14. ABSTRACT

During Year 3, results continued to accumulate that are consistent with the hypothesis that disfacilitation of wake-promoting systems by the hypocretin (Hcrt) receptor antagonist almorexant (ALM) results in less functional impairment than the inhibition of neural activity produced by the benzodiazepine receptor agonist zolpidem (ZOL). Measures of both spatial reference memory (Task 2a) and spatial working memory (Task 2b) in rodents treated with ALM were mostly indistinguishable from vehicle whereas impairments were clearly evident under ZOL. Similarly, wake-active Hcrt neurons can be recruited in the presence of ALM after sleep deprivation but not in the presence of ZOL (Task 3a). Conversely, although both drugs activate sleep-active cortical neurons, sleep-active cells are more strongly activated by ZOL (Task 4a). Lesions of the basal forebrain (BF), a wakefulness-promoting area, potentiated the hypnotic effect of ZOL without affecting the response to ALM (Task 3b), indicating different neural pathways underlie the actions of these two drugs. ALM promoted adenosine and glutamate release in the BF (Task 4b) whereas ZOL promoted GABA release, particularly during waking. An *In Vivo* Cellular Neurophysiology Laboratory was established to perform the electrophysiology and optogenetic experiments (Tasks 6a-c).

15. SUBJECT TERMS
Sleep, performance, drug, neurotransmitter, hypocretin, orexin, benzodiazepine, zolpidem, neurochemistry, microdialysis

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PROGRESS REPORT

"Effect of a Hypocretin/Orexin Antagonist on Neurocognitive Performance"

USAMRAA Grant W81XWH-09-2-0080

DR080789P1

Year 3: 8/1/11 to 7/31/12

Thomas S. Kilduff, Ph.D., Principal Investigator

INTRODUCTION

Almorexant (ALM) is a hypocretin/orexin (Hcrt) receptor antagonist with a novel mechanism of action that has shown promise as an effective hypnotic. Preclinical data demonstrate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. The overall hypothesis that underlies this research is that ALM produces less functional impairment than the benzodiazepine receptor agonist zolpidem (ZOL) because ZOL causes a general inhibition of neural activity whereas ALM specifically disfacilitates wake-promoting systems. Whereas the human study component will establish whether ALM is superior to ZOL in neurocognitive tests, the animal studies will compare the neural circuitry that underlies the activity of these compounds, their effects on sleep and performance, and the effects of these compounds on biomarkers associated with normal sleep.

BODY

Task 2. *Test the hypothesis that rodents receiving ZOL will show greater neurocognitive impairment than those receiving ALM or PBO.*

2a. Assessment of Almorexant effects on spatial reference memory in rats.
2b. Assessment of Almorexant effects on spatial working memory in rats.
2c. Assessment of Almorexant effects on psychomotor vigilance in rats.
2d. Synthesis of Almorexant.

Progress – Task 2a: All data collection for Task 2a is complete and most data analysis is complete. All water maze (WM) data for the effects of ALM on spatial reference memory are reported here. We have assessed the effects of ALM on spatial reference memory both following undisturbed and sleep deprivation conditions.

Methods: All rats were given a minimum of 3 wks for recovery from surgery and each rat was recorded for a 24 h period to determine basal sleep/wake patterns. Assessment the effects of ALM on spatial reference memory occurred on 2 consecutive days. On day 1, the acquisition of the task occurred in one session consisting of 8-12 consecutive WM trials with a 60 second (s) inter-trial interval. On the following day, rats were either left undisturbed and dosed with ALM, ZOL or vehicle (VEH) 6 h into their active period (ZT18) or kept awake for the first 6 h of the dark and then dosed. Subsequent to dosing, rats were left undisturbed for 90 min and then a retention probe trial was performed. For this test, the platform was removed from the WM and the rats were allowed to swim and search for the platform for 30 s. Parameters measured during the retention probe trial included the time and distance traveled in the quadrant of the WM where the platform had been on the acquisition day, as well as the latency and the number of entries into the target quadrant. EEG and EMG recordings were analyzed from the beginning of lights

out (ZT12) until initiation of the WM test (7.5 h later). For more details on our experimental procedures, please see the full protocols in our original proposal.

Results: As reported previously, both ALM (100 mg/kg i.p and p.o.) and ZOL (30 mg/kg i.p. and 100 mg/kg p.o.) had significant sleep-promoting effects. Waking (W) was decreased and non-rapid eye movement sleep (NR) increased by ALM and ZOL compared to vehicle. Although NR was increased to a greater extent following ZOL than ALM, rapid eye movement (REM) sleep was increased significantly more by ALM than by ZOL. Importantly, for the 60 min prior to WM testing, the ALM and ZOL groups of rats slept equivalent amounts; the differences between the sleep-promoting effects of ALM and ZOL occurred primarily during the first 30 min following drug administration. Confirming our previous findings, ZOL significantly reduced the latency to sleep onset compared to vehicle and ALM.

During the WM probe trial following undisturbed conditions, rats administered ZOL showed impairments in all parameters measured compared to rats administered VEH or ALM whereas ALM was indistinguishable from VEH for all measures (Figure 1). Following ZOL, rats swam significantly less (Fig. 1A), took longer to reach the target zone (Fig. 1C), spent less time in the target zone (Fig. 1E), and entered the target zone less frequently (Fig. 1G) compared to rats administered VEH or ALM.

During the WM probe trial following the 6 h sleep deprivation, ALM was again indistinguishable from VEH for all measures. Following ZOL, rats swam significantly less (Fig. 1B) and took longer to reach the target zone (Fig. 1D) compared to rats administered VEH or ALM. However, time spent in the target zone (Fig. 1F) and the frequency of entering the target zone (Fig. 1H) was not different from rats administered VEH or ALM.

These results are consistent with the hypothesis that, although both ALM and ZOL are effective hypnotic medications, rats would show less functional impairment following ALM than following ZOL treatment.

Progress – Task 2b: Similar to the assessment of the effects of ALM on spatial reference memory in Task 2a, we have proposed to assess the effects of ALM on spatial working memory following both undisturbed and sleep deprived conditions in Task 2b. All data collection is complete for the undisturbed condition and the data is reported here. For the sleep-deprived condition, all rats have been implanted and the tests are currently being completed.

Methods: The general protocol for assessing spatial working memory is as follows. Approximately one week before spatial working memory testing, rats are trained in a standard WM protocol as described in Task 2a. During this training, the location of the hidden platform remains constant across all trials. A baseline sleep recording occurs following training but prior to testing, in order to assess the undisturbed sleep-wake patterns of each rat. On the experimental day, rats are either left undisturbed prior to administration of drug at ZT18 or sleep deprived for 6h prior to drug administration. Rats are then left undisturbed until testing 60 min later.

The spatial working memory task consists of 6 pairs of trials. In the first trial, a cued platform marked with a flag is placed in one of 6 positions in the tank. Rats are released facing the wall from one of the 3 quadrants that does not contain the platform and are allowed 120 s to locate the cued platform before the researcher guides the rat to the platform. After 15 s on the

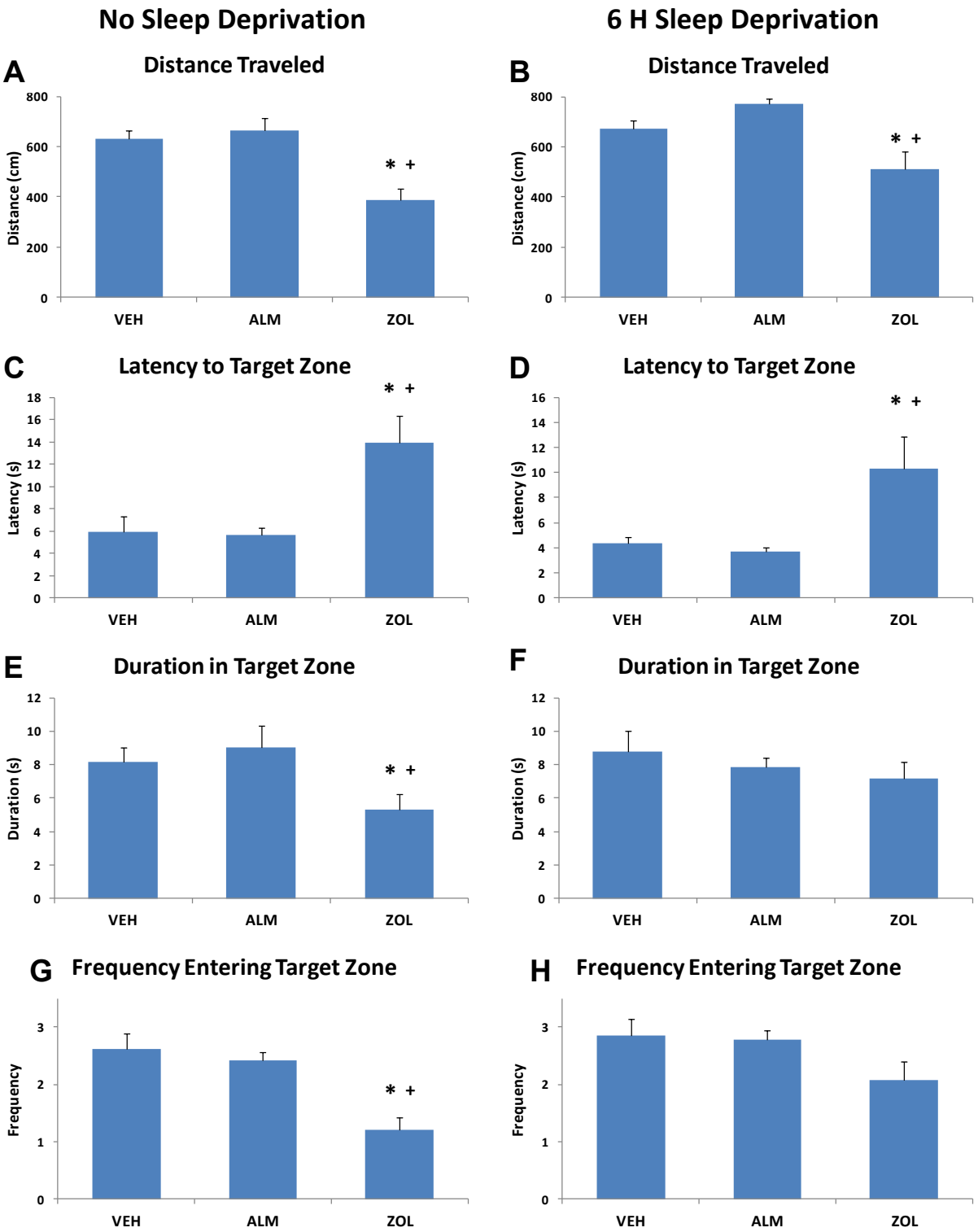

Figure 1. Measures of spatial reference memory during the WM probe trial. For the non-sleep deprived condition (left column), rats were left undisturbed for the first 6 h after lights off. Rats were then dosed and tested 90 min later. For the sleep deprived condition (right column), rats were kept awake for the first 6 h after lights off and then dosed. Following dosing, rats were left undisturbed and tested 90 min later. **A, B.** Distance traveled during the 30 s probe trial. Rats administered ZOL swam significantly less than rats administered vehicle or ALM following both undisturbed and sleep deprived conditions. **C, D.** Latency to

entry into the target zone. Rats administered ZOL took significantly longer time to enter the target zone compared to rats administered vehicle or ALM following both undisturbed and sleep deprived conditions. **E, F.** Time spent in the target zone. Rats administered ZOL spent significantly less time in the target zone compared to rats administered vehicle or ALM only following undisturbed conditions. **G, H.** Frequency of entry into the target zone. Rats administered ZOL entered the target zone significantly fewer times compared to rats administered vehicle or ALM only following undisturbed conditions.
* = significantly different from vehicle; **+** = significantly different from ALM.

platform, rats are removed from the WM and placed in a holding cage. The flag is then removed but the platform remains in the same location as in the first trial. Following a delay of 1, 5, or 10 min in the holding cage, the rat is placed back in the tank in one of the two quadrants that did not contain the platform and was not the starting quadrant from the first trial. Once the rats finds the platform, it is removed after approximately 5 s on the platform and placed back in a holding cage for 10 min before a new pair of trials with a novel platform location is given. The order of delays is counterbalanced so that each rat is tested twice at 1, 5, or 10 min delays between the cued and hidden platforms. Test measures are velocity, time and distance traveled to locate the platform during all tests.

Results: During the WM test trials following undisturbed conditions, rats administered ZOL showed impairments in almost all parameters measured compared to rats administered VEH or ALM whereas ALM was indistinguishable from VEH for all measures (Figure 2). Following ZOL, rats swam less (Fig. 2A), took longer to find the platform (Fig. 2B), and swam more slowly (Fig. 2C) compared to rats administered VEH or ALM. Importantly, following ZOL, rats failed to find the platform significantly more often than following ALM or VEH (Figure 3).

To investigate potential mechanisms underlying whether the platform was found or not, we analyzed distance traveled and velocity when the platform was found and when it was not found (Figure 4). During the few trials where the platform was not found following ALM or VEH, rats swam greater distances than when they found the platform (Fig. 4A vs. Fig. 4B), indicative of the searching behavior observed under these conditions. Following ZOL, however, distance traveled was not greatly different between the trials where the platform was located and when it was not. This measure indicates a relative lack of searching behavior in rats following ZOL treatment. In contrast, velocity did not differ for the trials when the platform was found compared to when it was not found for any drug condition. These results support our initial hypothesis that rats would perform more poorly (i.e., have greater functional impairment) following ZOL than following ALM. These results demonstrate that ALM impairs the performance of rats less than ZOL does in this spatial reference memory task.

Progress – Task 2c: The equipment for the rodent psychomotor vigilance (rPVT) test has been ordered the experiments are to begin immediately following the completion of the WM experiments. We expect to order rats and begin training by early September.

Progress – Task 2d: Synthesis of ALM. COMPLETED

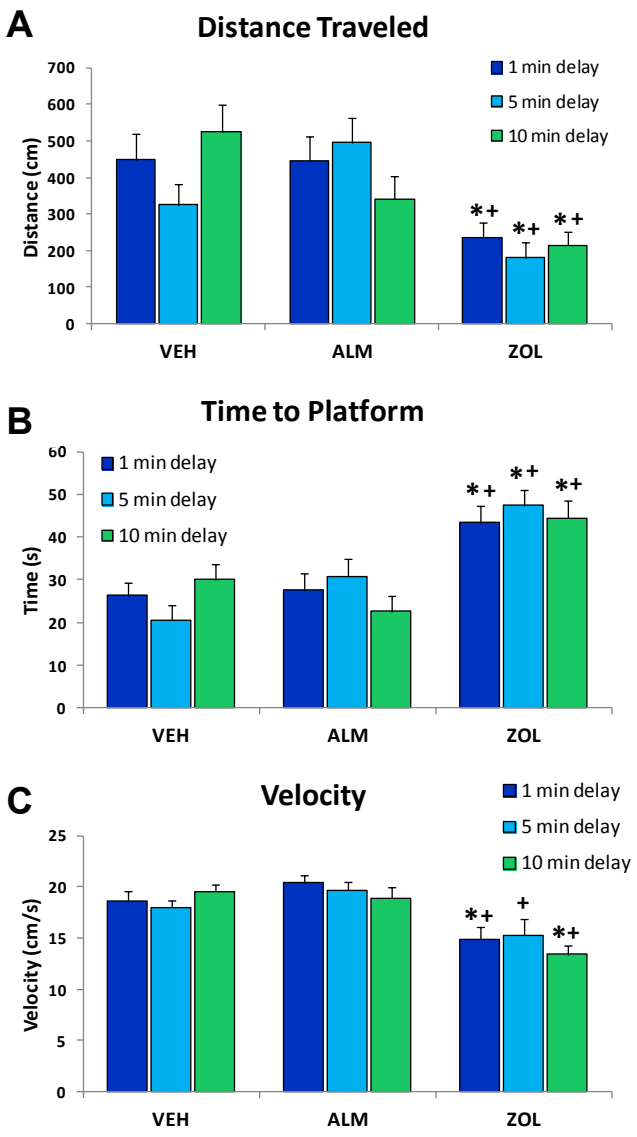

Figure 2. Measures of spatial working memory during the WM task. **A.** Distance traveled during the 60 s test trial. Rats administered ZOL swam significantly less than rats administered vehicle or ALM. **B.** Time taken to find platform during the 60 s test trial. Rats administered ZOL took significantly longer to find the platform than rats administered vehicle or ALM. **C.** Velocity of the rat during the 60 s test trial. Rats administered ZOL swam significantly slower than rats administered vehicle or ALM.
* = significantly different from vehicle;
+ = significantly different from ALM.

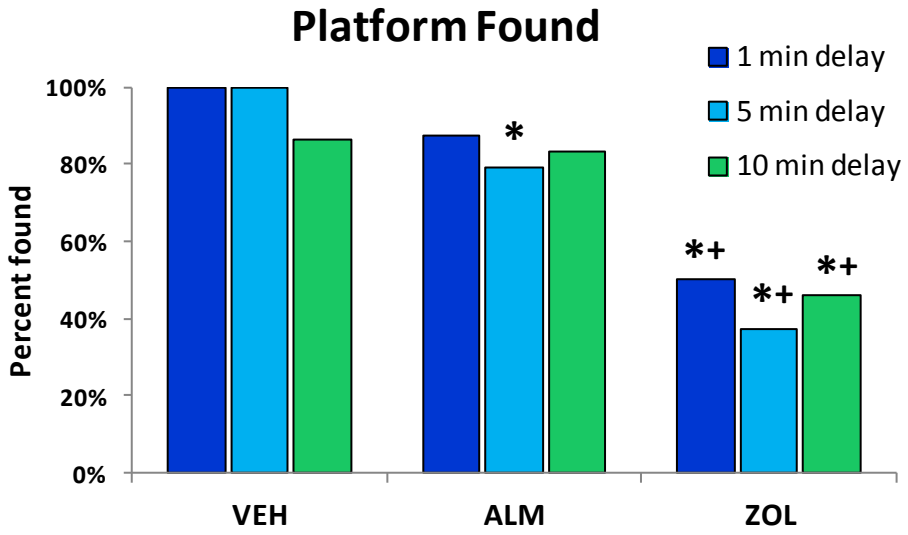

Figure 3. Percentage of trials during which rats found the platform during the WM test trial measuring spatial working memory. Rats administered ZOL found the platform significantly less than rats administered vehicle or ALM. * = significantly different from vehicle; **+** = significantly different from ALM.

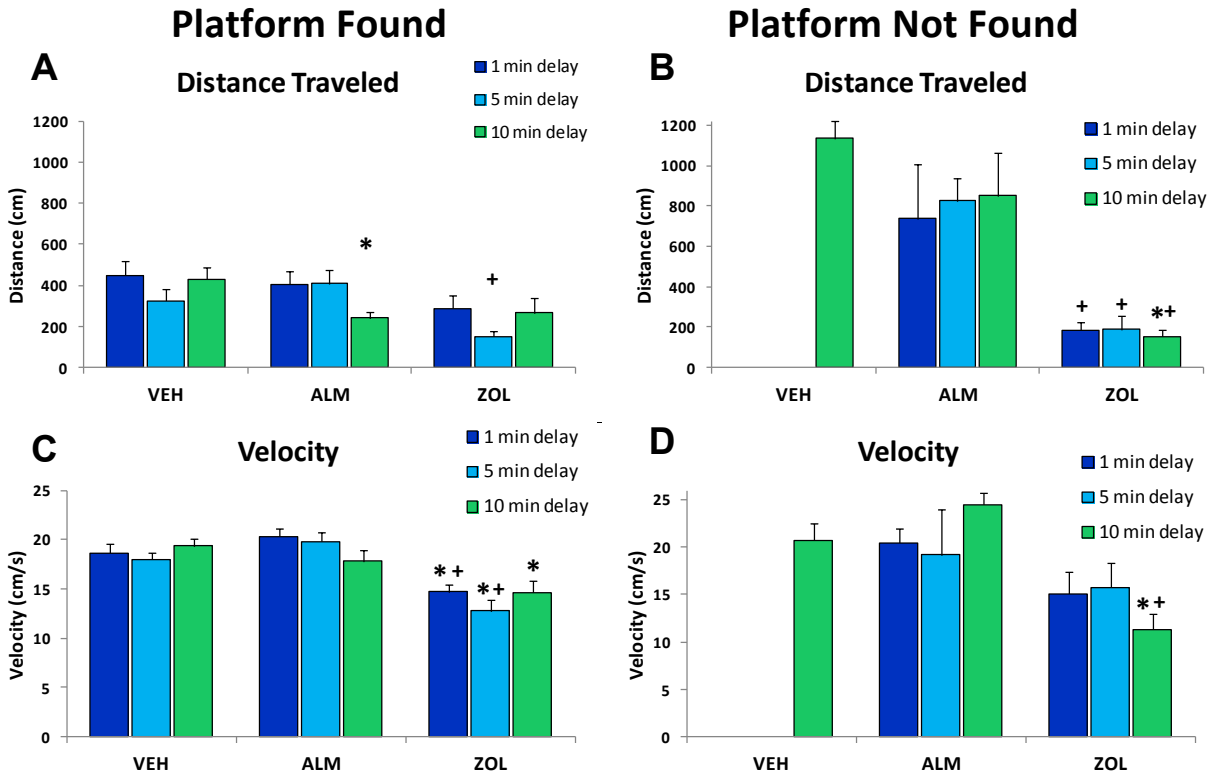

Figure 4. Distance traveled and velocity of the rats during the spatial working memory WM task separated by whether the platform was found (left panels) or not found (right panels) during the trial. **A, B.** Distance traveled during the 60 s test trial. Rats administered VEH or ALM swam further during the trials when the platform was not found than when it was found. Rats administered ZOL swam similar distances whether the platform was found or not. **C, D.** Velocity during the 60 s test trial. The velocity of the rats was similar whether the platform was found or not during all three drug conditions. * = significantly different from vehicle; **+** = significantly different from ALM.

Task 3. *Test the hypothesis that the Hcrt antagonist ALM induces sleep by selectively disfacilitating the activity of the histaminergic, serotonergic, noradrenergic and cholinergic wake-promoting systems whereas the BzRA ZOL causes a generalized inhibition of the brain.*
3a. Double-label immunohistochemistry with Fos and phenotypic markers.
3b. Assessment of hypnotic efficacy in saporin-lesioned rats.
3c. Assessment of hypnotic efficacy in transgenic mice.

Progress - Task 3a: As described in the last Progress Report, we generated a cohort of animals dosed intraperitoneally with 100 mg/kg ALM, 30 mg/kg ZOL, or VEH for histological studies. This year we processed 46 additional rats dosed with 1 ml of 100 mg/kg ALM p.o., 1 ml of 100 mg/kg ZOL p.o. or 1 ml of VEH. This dosing regimen was adapted to match the dosage and delivery used for the behavioral studies in Task 2.

To assess the influence of ALM and ZOL on the activity of sleep/wake regulatory neurons, we performed an immunohistochemical study using c-Fos as a marker of neuronal activity. Rats were administered ALM (100 mg/kg p.o., n=16), ZOL (100 mg/kg p.o., n=16), or vehicle (n=14) at ZT18. Half of the animals in each drug treatment condition were allowed to sleep for 1.5h after dosing, whereas the remaining rats were sleep deprived by gentle handling (SD). All animals were then deeply anesthetized, perfused and the brains sectioned. Double-label immunohistochemistry for Fos, a marker of functional activity, and the neuropeptide hypocretin (Hcrt; a "wake-active" hypothalamic neuronal population) was performed in coronal brain sections at the level of the lateral hypothalamus. The results confirmed the main findings reported in the last Progress Report: wake-active Hcrt neurons showed higher levels of Fos expression after SD than after the undisturbed condition only in the ALM-treated rats (Fig. 5) whereas there was no such difference for the ZOL-treated rats. These results indicate that activation of the Hcrt neurons by SD is unimpaired in the presence of ALM whereas ZOL appears to block such activation.

To assess Fos expression in other populations of wake-active neurons, we developed staining protocols for immunohistochemical staining of adenosine deaminase, serotonin, dopamine beta-hydroxylase, and choline acetyltransferase. During Year 4, double immunochemistry for Fos and these markers will be carried out using the tissue already processed as described above.

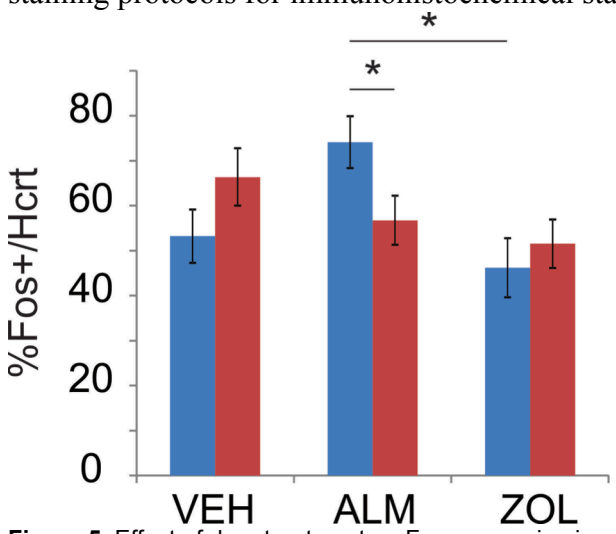

Figure 5. Effect of drug treatment on Fos expression in wake-active Hcrt neurons. Blue bars depict group means for sleep deprived rats, red bars for undisturbed rats. * = p< 0.05 for pairwise comparisons following ANOVA.

Progress - Task 3b: To test the hypothesis that ALM induces sleep by selectively disfacilitating the activity of cholinergic wake-promoting systems, 16 male Sprague-Dawley rats were instrumented for telemetric recording of EEG, EMG, locomotor activity (LMA) and core body temperature (CBT), and were injected bilaterally in the basal forebrain with neurotoxin 192-IgG-saporin (BFx; n=8) or sterile saline (Sham; n=8). Injections were delivered stereotaxically via a calibrated glass micropipette using a pressurized air delivery system (Picospritzer, Parker-Hannefin). Rats were given 3 wk recovery to ensure that neuronal degeneration was complete. One BFx rat was removed from the study due to postoperative weight loss (final n=7). Prior to dosing, rats were recorded for a 24 h undisturbed baseline, sleep-deprived for 6 h starting at lights-off, and then allowed to recover undisturbed. After an 18 h recovery period, rats were given p.o. ALM 30, 100 and 300 mg/kg, ZOL 10, 30 and 100 mg/kg, and VEH at lights-off (ZT 12) in balanced order with at least 3 d between treatments. EEG, EMG, LMA and CBT were recorded continuously throughout experimentation.

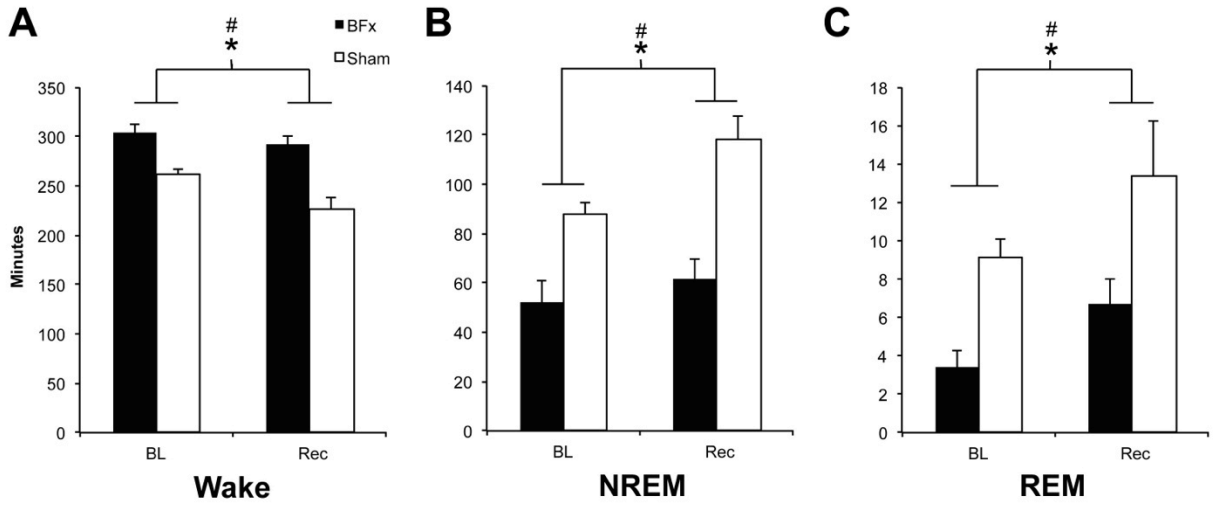

Figure 6. Cumulative Wake, NREM and REM sleep time in minutes for BF-lesioned (black) and Sham-operated (white) rats at baseline and after 6 h sleep deprivation (SD) in the dark phase. Bars represent total time spent in state from ZT 18 - ZT 23. *, significant main effect of lesion; #, significant main effect of SD. All effects considered significant at $p < 0.05$.

BFx decreased NREM and REM sleep and increased waking in the dark phase compared to Sham at baseline and following 6 h SD (Fig 6). By contrast, baseline sleep in the light phase was unaffected (not shown). While this stimulatory effect is at odds with the BF's proposed role as an arousal-promoting system, the BF is also known to mediate homeostatic increases in sleep, such as following prolonged wakefulness. Thus, the lesions, by destroying an important site of integration for homeostatic sleep cues, likely decreased the ability to respond to elevated sleep pressure during their normal active phase.

To control for reduced baseline sleep in BFx animals compared to controls, sleep from the drug dosing regimen was normalized to each individual's vehicle condition. ALM and ZOL increased NREM sleep over VEH as expected, but did so differently in BFx and Sham rats (significant drug x lesion x ZT interaction, $F_{30,390} = 2.94$, $p < 0.0001$; Fig. 7B-D). ALM increased NREM sleep to a similar extent in BFx and Sham rats (150% - 200% of vehicle), whereas ZOL induced substantially larger increases in NREM sleep in BFx (300% - 450%) than in Sham rats (150% - 200%; Fig. 7E-G). BFx rats had fewer NREM bouts than Shams when given vehicle and ALM, but similar bout numbers when given ZOL. NREM bout length was unaffected by

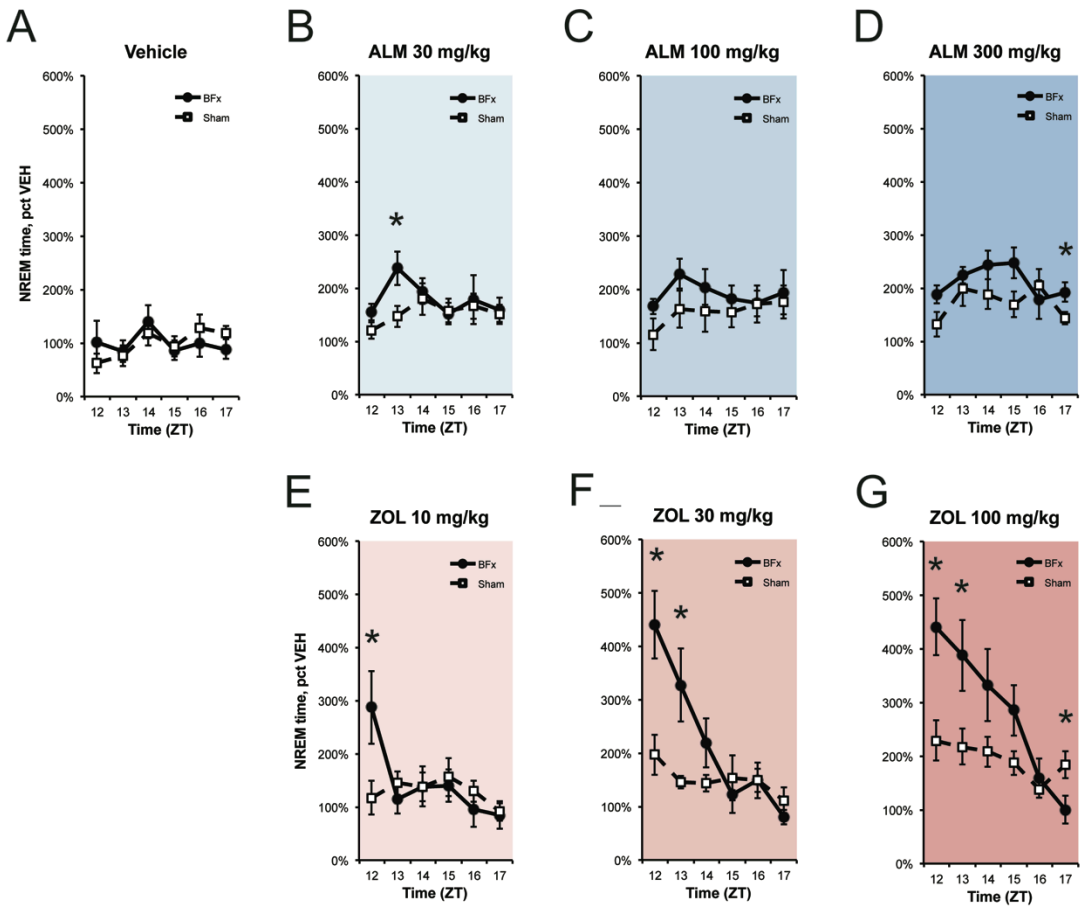

Figure 7. Hourly NREM sleep time in BFx (black) and Sham-operated (white) rats for 6 h following administration of VEH(A), ALM (B-D, blue bkgd) and ZOL (E-G, red bkgd) at lights-out (ZT12). For each individual, NREM time per h was normalized to the 6h-mean NREM time for VEH. Asterisks indicate significant effect of lesion; all effects considered significant at $p<0.05$.

ALM, but was increased by ZOL; interestingly, this increase was smaller in BFx rats than in Shams. REM sleep was increased by ALM but not ZOL, and BF lesions did not alter the response to either drug. These data suggest that decreased cholinergic tone in BFx rats may make them hypersensitive to ZOL without affecting the response to ALM.

In pilot studies, we verified our microinjection technique and the dosage for lesioning the wake-promoting noradrenergic locus coeruleus (LC) using anti-DBH-saporin (DBH-SAP). DBH-SAP was infused into the 3rd ventricle using a Hamilton syringe with a 35-gauge stainless steel needle coupled to a digital microinjection pump (World Precision Instruments, Sarasota FL). Infusions of 2.5 µg – 5 µg DBH-SAP effectively lesioned the LC (Fig. 8B), whereas infusions of 3 µg 192-IgG-SAP (Fig. 8A) or sterile water (not shown) left the LC intact. EEG implant and lesion surgery is underway, with behavioral assessment and drug response to follow. Hcrt-SAP, which we proposed to use to lesion the histaminergic TMN, is no longer commercially available. We are investigating possible alternate sources of this compound.

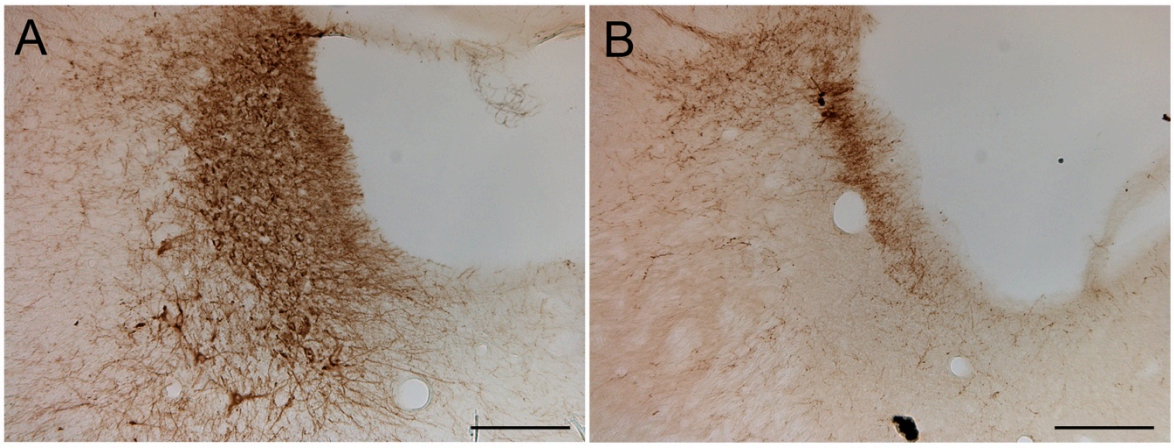

Figure 8. DBH-immunoreactivity in the locus coeruleus (LC) of rats injected with the selective neurotoxins 192-IgG saporin, which targets cholinergic neurons in the basal forebrain (A), or DBH-saporin, which targets noradrenergic neurons (B). Scale bar = 200 µm.

Progress - Task 3c: We identified an alternate source for *Pet1-Lmx1b(ffp)* mice; we are now awaiting importation of these mice from the University of Iowa, and *Dbh* KO mice from the University of Pennsylvania.

Task 4. *Test the hypothesis that ALM, but not ZOL, induces sleep by facilitating the mechanisms that underlie the transition to normal sleep.*
4a. Effects of ALM and ZOL on sleep-active brain areas.
4b. BF adenosine (ADO) release in response to oral ALM and ZOL.
4c. BF adenosine (ADO) release in response to ALM and ZOL by dialysis.

Progress - Task 4a: To match the doses and drug delivery route used in the Aim 2 experiments, we processed 46 more rats for histological studies as described for Task 3a. Double immunohistochemistry for the marker of functional activity, Fos, and the enzyme neuronal nitric oxide synthase (nNOS), a marker for sleep-active cortical neurons, was performed in coronal sections at the level of the anterior commissure. The results confirmed the data obtained from different drug dosing regimens reported in the last Progress Report. SD inhibited Fos expression in these sleep-active neurons in all drug conditions (Fig. 9). If the rats were allowed to sleep, a significantly higher proportion of cortical nNOS neurons expressed Fos for the groups dosed with either ALM or ZOL. This result indicates that neither ALM or ZOL directly activate cortical nNOS neurons, rather, activation of cortical nNOS neurons is permitted by sleep, which is promoted by both compounds. We also found that a smaller proportion of cortical nNOS neurons expressed Fos in animals dosed with ALM than in animals dosed with ZOL. This might be

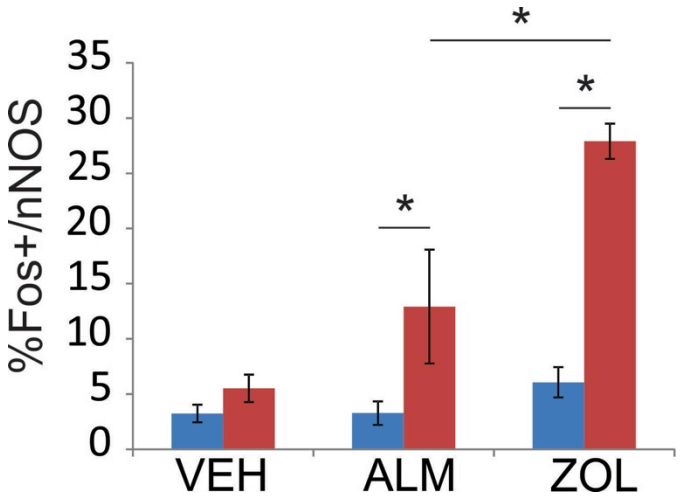

Figure 9. Effect of drug treatment on Fos-expression in sleep-active cortical nNOS neurons. Blue bars depict group means for sleep deprived rats, red bars for undisturbed rats. * = p< 0.05 for pairwise comparisons following ANOVA.

due to the delayed sleep onset induced by ALM relative to ZOL, resulting in a smaller amount of cumulative sleep time in the 1.5 h before perfusion.

To test whether there is a dose-response effect of ZOL-induced activation of cortical nNOS neurons, we added a group of 8 rats that were dosed with 30 mg/kg ZOL p.o. and again either sleep-deprived or left undisturbed for 1.5h until perfusion. Figure 10 compares the effects of this dose with the 100 mg/kg ZOL p.o. and VEH as described above. In contrast to the higher dose, 30 mg/kg ZOL p.o. did not increase the activation of sleep-active cortical nNOS neurons significantly, indicating a dose-response relationship between ZOL and activation of this neuronal population.

To test whether sleep pressure would influence the efficacy of ZOL to induce Fos in cortical nNOS neurons, we performed an additional experiment in 21 rats. The rats were dosed with either 100 mg/kg p.o. ZOL or VEH at ZT 12, when sleep pressure naturally is low. Half of the rats were sleep deprived for 6 h before dosing to increase sleep pressure. Two hours after dosing, all rats were perfused, brains were processed, and immunohistological double-labeling for Fos and nNOS was performed in coronal sections at the level of the anterior commissure. For both vehicle and ZOL-treated rats, the proportion of Fos-expressing cortical nNOS neurons was significantly higher when the rats had increased sleep pressure (Fig. 11). This result is consistent with our results presented in Figure 9 that ZOL is permissive for activation of cortical nNOS neurons but does not activate them directly, rather, sleep pressure is needed for direct activation.

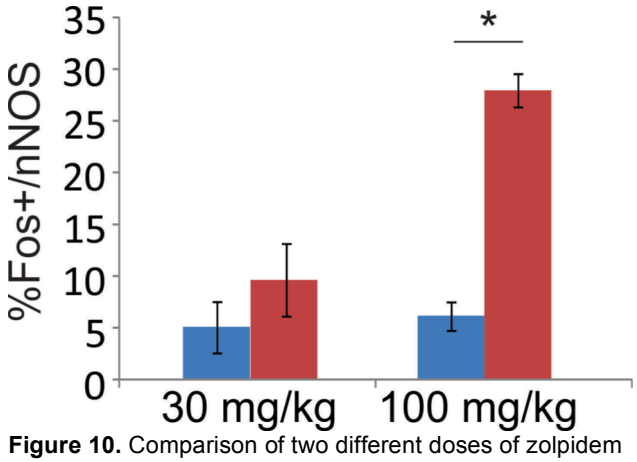

Figure 10. Comparison of two different doses of zolpidem on Fos-expression in sleep-active cortical nNOS neurons. Blue bars depict group means for sleep deprived rats, red bars for undisturbed rats. * = p< 0.05 for pairwise comparisons following ANOVA.

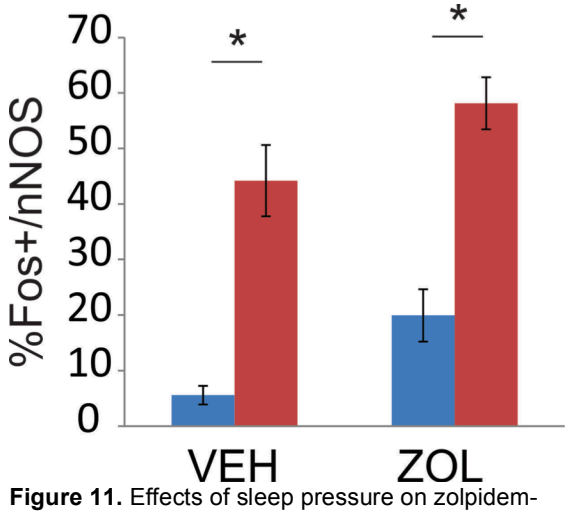

Figure 11. Effects of sleep pressure on zolpidem-induced Fos expression in sleep-active cortical nNOS neurons. Blue bars depict group means for animals with low sleep pressure, red bars for high sleep pressure. * = p< 0.05 for pairwise comparisons following ANOVA.

Progress – Task 4b: Task 4B was undertaken to study the effects of oral ALM and ZOL on basal forebrain (BF) adenosine (ADO) release, glutamate (GLU), and GABA release during sleep and wakefulness. We tested the hypothesis that oral ALM induces sleep by facilitating the mechanisms that underlie the transition to normal sleep. In contrast to ZOL, which affects $GABA_A$ receptors that are widely distributed in the CNS, we hypothesize that ALM acts through blockade of post-synaptic Hcrt receptors, thereby disfacilitating excitation in the BF. We used *in vivo* microdialysis and HPLC analyses to examine BF glutamate, GABA, and ADO efflux following oral ZOL (10 mg/kg), ALM (100 mg/kg), or placebo (VEH) combined with behavioral sleep analyses.

Experimental design. Male Sprague-Dawley rats were implanted with chronic recording devices (F40-EET, Data Sciences Inc., St Paul, MN) for continuous recordings of EEG, EMG, CBT, and LMA via telemetry as described previously (Morairty et al., 2008). Rats were then placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA) for surgical implantation of a unilateral, stainless steel 26-gauge guide cannula aimed at the BF for microdialysis recovery of ADO, glutamate, and GABA. BF coordinates relative to bregma were P -0.3, L +2.0, V –5.0 (Paxinos and Watson, 2009).

Animals were given a 2-3 wk post-surgical recovery period prior to entering the experimental paradigm, conditioned to the microdialysis chamber, and handled for at least 30 min every day for 1 wk prior to the onset of the experiment to limit stress on the day of the experiment from exposure to a novel environment. A microdialysis probe was inserted through the guide cannula 16 h prior to the onset of the experiment day and continuously perfused with aCSF. At the start of the experiment (4.5 h into the dark period, ZT16.5), three 30 min baseline samples (1 µL/min flow rate, 30 µL TV) were collected from freely-moving animals to assess basal levels of ADO, GLU, and GABA while baseline EEG, EMG, T_b and LMA were collected to assess behavior. Each rat received one treatment in random order (washout period minimum 1 week) with parallel microdialysis sampling of the BF. Drug doses included ALM (100 mg/kg), ZOL (10 mg/kg) and VEH. One of three drugs was subsequently given p.o. to the animals 6h into the dark period (the rats' normal active period) (ZT18), and six additional 30 min samples were collected to assess the effects of the drug on behavior and neurotransmitter release in the BF. Behavioral measures were simultaneously collected for an additional 1.5 h (total 12 h) post-microdialysis. All samples were collected at 4ºC and immediately stored at -80ºC until processed for ADO by HPLC/UV and AA/GABA by HPLC-EC detection.

Behavioral state analyses. Following completion of data collection, sleep-wakefulness was scored in 10 s epochs by examining the recordings visually using Neuroscore software (Data Sciences Inc., St Paul, MN). Any epochs that contained recording artifacts were tagged and excluded from subsequent analyses. EEG and EMG data were scored for waking (W), rapid eye movement (REM), and non-REM (NR) sleep. CBT and LMA (counts per minute) were analyzed as hourly means. Individual state data were analyzed as time spent in each state (W, REM, and NR) per hour. Latency to NR and REM onset for each rat was calculated from the time of drug injection. To assess any pharmacological effects on the consolidation of behavioral states, cumulative time spent in W, NR, and REM and the duration and number of bouts of each state was calculated for 3 h following drug administration relative to each 30 min dialysis sample obtained pre- and post-drug administration. Descriptive statistics and analysis of variance (ANOVA) analyses were performed on all behavioral measures. Where ANOVA indicated a probability (P) value < 0.05, Dunnett's *post hoc* was used to determine significance between groups.

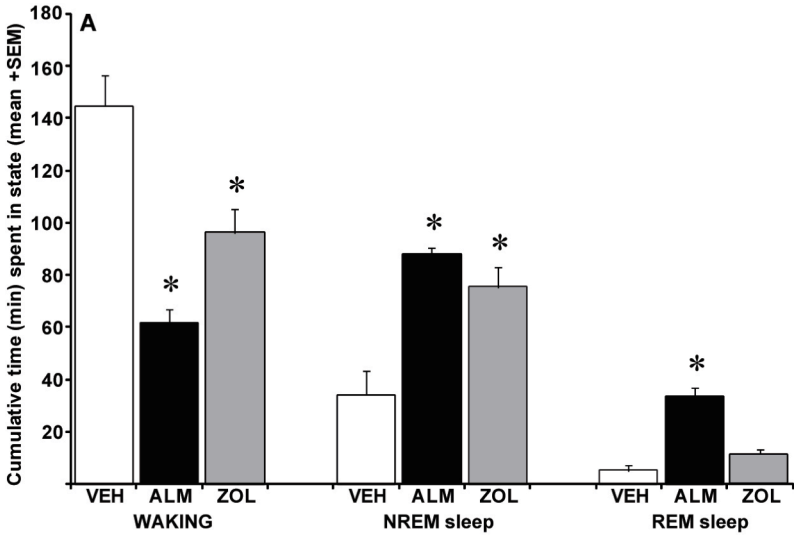

Figure 12. Time each behavioral state for VEH, ALM-, and ZOL-treated rats. Values (means ± SEM) are for a 3 h period during the dark phase. *p<0.05 for each treatment was determined by one-way ANOVA with Tukey's multiple comparison *post hoc* analyses.

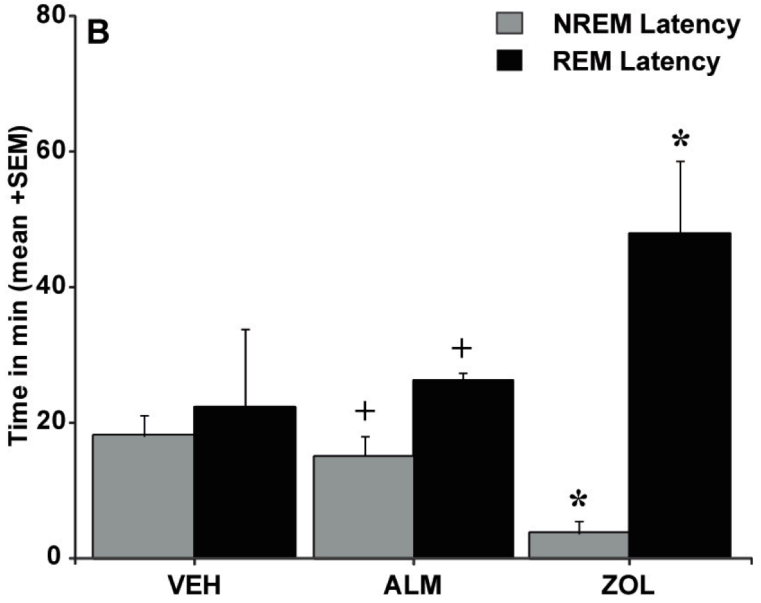

Figure 13. Latency to the onset of NREM and REM sleep following oral administration of ALM as compared to ZOL. * and + p<0.05 demonstrate significant differences from VEH (*) or from drug treatment (+).

HPLC Analyses. All microdialysis samples were split (10 µL for ADO, 20 µL for AA/GABA) into two vials for HPLC analyses. ADO samples were separated by reverse-phase HPLC with a Kinetic column (Phenomenex C18 150 x 4.6mm) and monitored at 254 nM by UV. The mobile phase consisted of 10 mM Na_2HPO_4 (pH = 4.5), and 7% acetonitrile and was set to a flow rate of 0.8 mL/min. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex, Corp). Amino acids, glutamate and GABA were assayed using HPLC-EC. The mobile phase consisted of 100

mM Na_2HPO_4, 22% MEOH, and 3.5% acetonitrile, pH 6.75 and set to a flow rate of 0.4 mL/min. The amino acids were detected by precolumn derivitization using O-phtaladehyde (OPA) and 2-mercaptoethanol (βME) with automation at 4°C, 2 min prior to injection into the HPLC. Separation was achieved with a reversed-phase column by Shiseido (Capcell Pak C18, 3.0 mm ID x 75 mm, 3 μm) and electrically detected at the following potentials; E1; +150 mV, E2; +550 mV, Guard +600 mV. Calibration curves were constructed using Chromeleon 6.8.0 software (Dionex Corp). Descriptive statistics and a two-way ANOVA were used to determine the effect of sleep-wake states on ADO, glutamate, and GABA release. Post hoc comparisons were performed using Tukey's multiple pairwise comparison tests. A probability (*P*) value < 0.05 was used to evaluate the significance of all statistical tests.

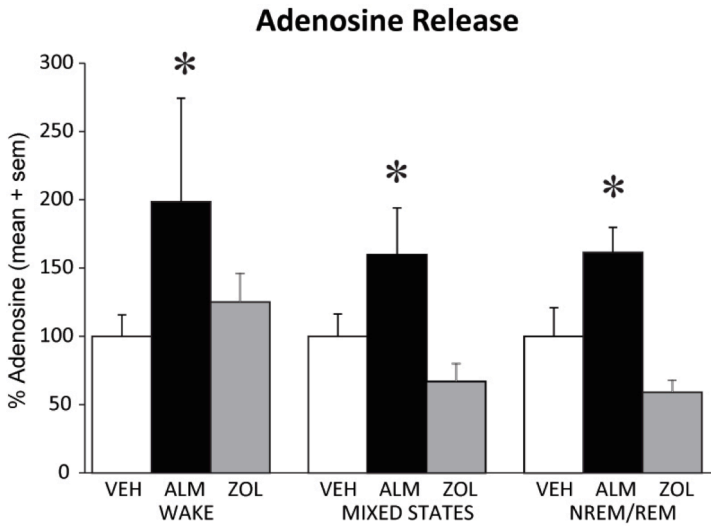

Figure 14. ADO release in the BF increased (*p<0.05) when sampled during all conditions following ALM (p.o.).

Results. To date, we have now completed recording, sampling and analysis of the proposed 8 rats per treatment group in Task 4B. Preliminary analyses were presented at the Society for Neuroscience meeting, Washington D.C. in November of 2011. As illustrated in Figure 12, the results of the effects of the various drugs on sleep-wake behavior demonstrate that ALM significantly promotes the amount of time spent in NREM and REM sleep as has been previously described (Dugovic et al., 2009). In addition, ALM significantly alters the latency to the onset of NREM and REM sleep compared to ZOL and VEH (Figure 13).

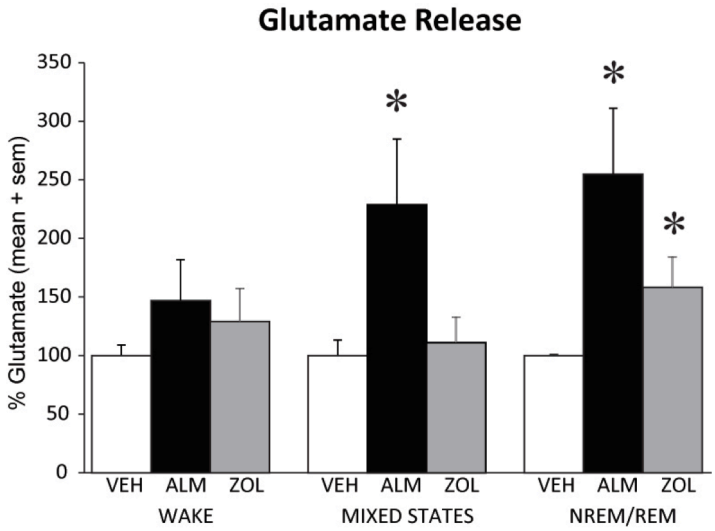

Figure 15. Glutamate release in the BF significantly increased after dosing with ALM when sampled during mixed states and NREM/REM compared to VEH (*p<0.05).

Dialysis samples were split into two and processed for both ADO and glutamate/GABA content. Two-way ANOVA revealed a significant drug x state interaction for all neurotransmitters. Tukey's *post hoc* comparisons (*p<0.05) were applied to determine significant differences between conditions.

The effects of the hypocretin antagonist ALM shows that this compound promotes adenosine (ADO) release in the basal forebrain (BF) during behavioral states of wakefulness, sleep, and mixed sleep-wake states (Figure 14). While ALM appears to promote ADO release in the BF during Wake, ZOL promotes

GABA release (Figure 16). During Mixed States, which involve transitions between Wake and Sleep, ALM also promotes GLU release (Figure 15). During predominantly NREM/REM sleep, both ALM and ZOL enhance GABA and GLU release (Figures 15 and 16).

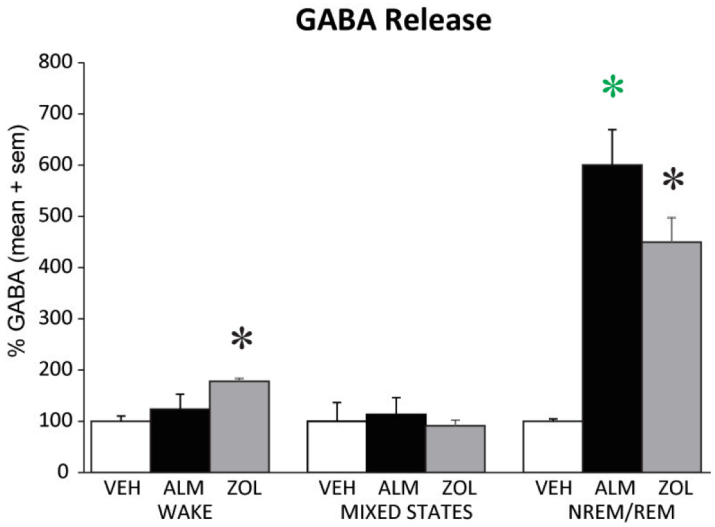

Figure 16. GABA release in the BF was significantly higher after dosing with ALM and ZOL during NREM/REM compared to VEH (*p<0.05).

As a result of positive feedback surrounding these data that were presented at the Society for Neuroscience, we decided to add an additional group of animals to the study aims to evaluate how ALM and ZOL's effects would alter neurotransmitter release under conditions of constant wakefulness.

Experimental design. Male Sprague-Dawley rats were implanted with chronic recording devices and cannulae directed at the BF as described above. Animals were given a 2-3 wk post-surgical recovery period prior to entering the experimental paradigm, conditioned to the microdialysis chamber, and handled for at least 30 min every day for 1 wk prior to the onset of the experiment to limit stress on the day of the experiment from exposure to a novel environment. A microdialysis probe was inserted through the guide cannula 16 h prior to the onset of the experiment day and continuously perfused with aCSF. We performed six hours of sleep deprivation (constant wakefulness) and permitted two hours of recovery sleep. At the start of the experiment (4.5 hours into the dark period, ZT16.5), four 30 min baseline samples (1 µL/min flow rate, 30 µL TV) were collected from freely-moving animals to assess basal levels of ADO, GLU, and GABA and baseline EEG, EMG, were collected to assess constant wakefulness. Each rat received one treatment in random order (washout period minimum 1 week) with parallel microdialysis sampling of the BF. Drug doses included ALM (100 mg/kg), ZOL (10 mg/kg) and VEH. One of three drugs was administered (p.o.) to the animals 6h into the dark period (ZT18), and nine 30 min samples were collected to assess the effects of the drug on constant wakefulness and neurotransmitter release in the BF. Additionally, four samples were collected for an additional two hours and the rats were allowed recovery sleep. All samples were collected at 4ºC and

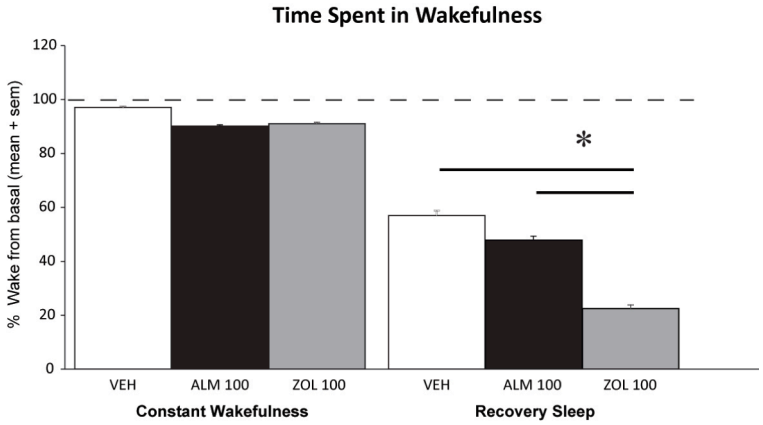

Figure 17. Percent time spent in wakefulness and recovery sleep following VEH, ZOL, and ALM treatment. *p<0.05 for each treatment was determined by one-way ANOVA with Tukey's multiple comparison *post hoc* analyses.

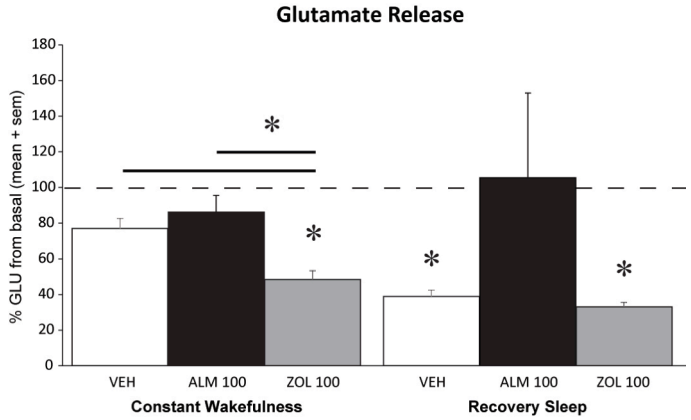

Figure 18. GLU release in the BF is not affected by ALM during waking and RS. ZOL caused a decrease in GLU release during wakefulness that persists into RS. *p<0.05 for each treatment was determined by one-way ANOVA with Tukey's multiple comparison *post hoc* analyses.

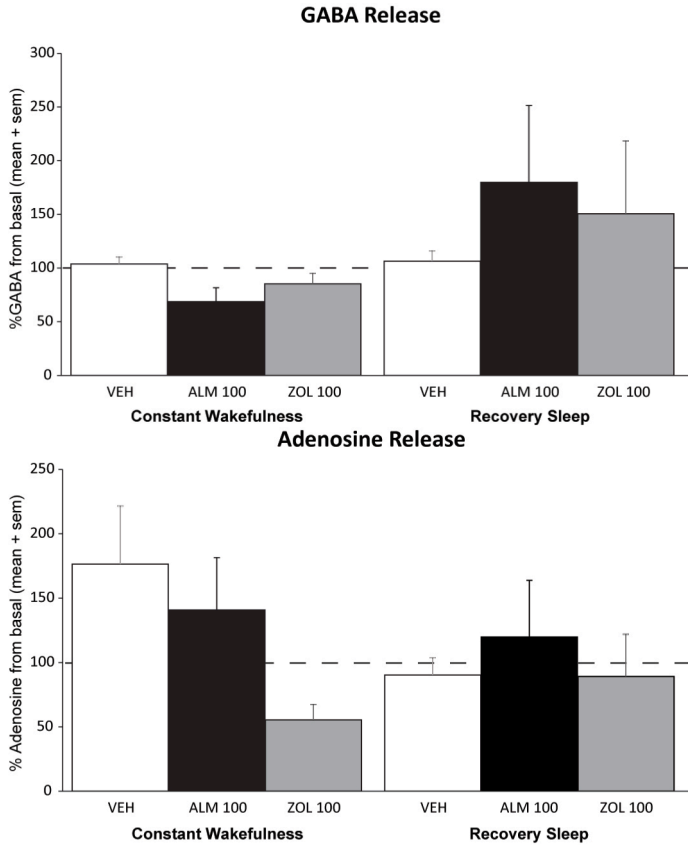

Figure 19. GABA and ADO release in the BF does not appear to be affected by either oral ALM or ZOL during constant waking or recovery sleep.

immediately stored at -80ºC until processed for ADO by HPLC/UV and AA/GABA by HPLC-EC detection.

Figure 17 summarizes the effects of VEH, ZOL, and ALM administration (p.o.) on constant wakefulness and recovery sleep for each treatment condition following drug delivery.

Analyses of neurotransmitter levels demonstrate GLU release in the BF is not altered by ALM when delivered during conditions of constant wakefulness or when the animals are permitted recovery sleep (*p<0.05; Tukey's *post hoc* test). However, oral ZOL significantly decreases BF GLU during constant waking that persists into recovery sleep (Figure 18; *p<0.05).

Preliminary results of GABA and ADO levels suggest that these neurotransmitter systems are not affected by drug administration under conditions of constant wakefulness and values appear to be near basal release levels upon recovery sleep (Figure 19). These neurotransmitter results provide additional evidence for dynamic neurochemical changes underlying Hcrt modulation of sleep-wakefulness.

All of these behavioral and neurotransmitter analyses will be submitted for publication once statistical power has been achieved with the appropriate number of animals per treatment group for constant wakefulness and recovery sleep. The originally proposed study design for Task 4C, BF ADO release in response ALM and ZOL by local dialysis, is not feasible as neither ALM nor ZOL readily pass across the dialysis membrane (testing in-house). Thus, we propose to continue evaluating ALM and ZOL effects on neurotransmitter levels in the BF under conditions of constant wakefulness and subsequent recovery sleep.

Task 6: *Utilize optogenetics and in vivo physiology to compare the neural circuitry underlying ALM-induced vs. ZOL-induced sleep.*

6a. Determine whether activation of the Hcrt system is sufficient to induce arousal in the presence of ALM vs. ZOL.

6b. Determine whether ALM affects the activity of subcortical sites downstream from the Hcrt neurons.

6c. Determine how ALM and ZOL affect the activity of cortical neurons.

Progress - Task 6: In order to achieve the goals proposed in Aim 6, it was necessary to build an *In Vivo* Cellular Neurophysiology Laboratory with the capabilities to perform the electrophysiology and optogenetic experiments proposed in Tasks 6a-c. Optogenetics experiments require that the animal under study expresses the Channelrodopsin-2 (ChR2) protein in the desired neuronal population, in our case, the HCRT neurons in the lateral hypothalamus. We chose the transgenic instead of the viral approach to achieve this, i.e., to breed a transgenic mouse line that expresses this light-sensitive ion channel only in HCRT neurons because it allows the manipulation of larger amounts of neurons and fortunately our collaborator in Japan, Dr. Akihiro Yamanaka has developed such a transgenic mouse and he provided us not only with the mice, but also he trained the postdoctoral fellow Dr. Jaime Heiss on the procedures to perform fiber optic implants in the lateral hypothalamus followed by tethered EEG/EMG recordings with optogenetic stimulation. Additionally, our existing *In Vitro* Cellular Neurophysiology Laboratory had to be upgraded in order to perform *in vitro* validation of the transgenic mice and optimization of the stimulation protocol by directly observing how single neurons are affected by the light stimulation.

Since the change in the Statement of Work and associated rebudgeting was approved in November, 2011, we have successfully built an *in vivo* lab that incorporates intracellular and

Table 1. Equipment acquired for the *In Vivo* Cellular Neurophysiology Laboratory

Instrument	Supplier	Model	Quantity
Vibration isolation table	TMC	63-544	1
Microelectrode amplifier	Molecular Devices	Multiclamp 700B	1
Data acquisition system	Molecular Devices	Digidata 1440	1
Light engine	Lumencor	Spectra 3	2
Oscilloscope	Hitachi	V-1565	1
Extracellular data acquisition system	Tucker-Davis Tech.	RZ2-4	1
96-channel preamplifier	Tucker-Davis Tech.	PZ2-96	1
Low impedance headstage	Tucker-Davis Tech.	RA16LI-D	1
Data streamer	Tucker-Davis Tech.	RS4-1	1
Implantable rodent headstage	Tucker-Davis Tech.	32-Channel ZIF-Clip	2
External battery	Tucker-Davis Tech.	PZ-BAT	1
Microwire array	Tucker-Davis Tech.	32 Channel ZIF-Clip	20
Microwire array	Tucker-Davis Tech.	16 Channel ZIF-Clip	20
4-axis micromanipulator	Siskiyou, Inc.	MX7600	2
Surgical microscope	Leica Microsystems	M320FL	1
Animal temperature controller	WPI, Inc.	ATC1000	1
Micro drill	WPI, Inc.	Omnidrill 35	1

extracellular recording systems with two computer-controlled light engines that enables us to successfully record intracellular and extracellular neuronal activity, local field potentials, EEG, and EMG while delivering light into the brain through an optical fiber. Table 1 provides a list of the equipment that has been installed in the *In Vivo* Cellular Neurophysiology Laboratory. This effort has also required an extensive amount of time to gain familiarity with the hardware and with the more than 10 different programs associated with it (including custom programing languages) and the inevitable fabrication of custom cables and miscellaneous other infrastructural adaptations. Figure 20 provides an example of one of our first recordings conducted in our new *In Vivo* Cellular Neurophysiology Laboratory from an awake mouse.

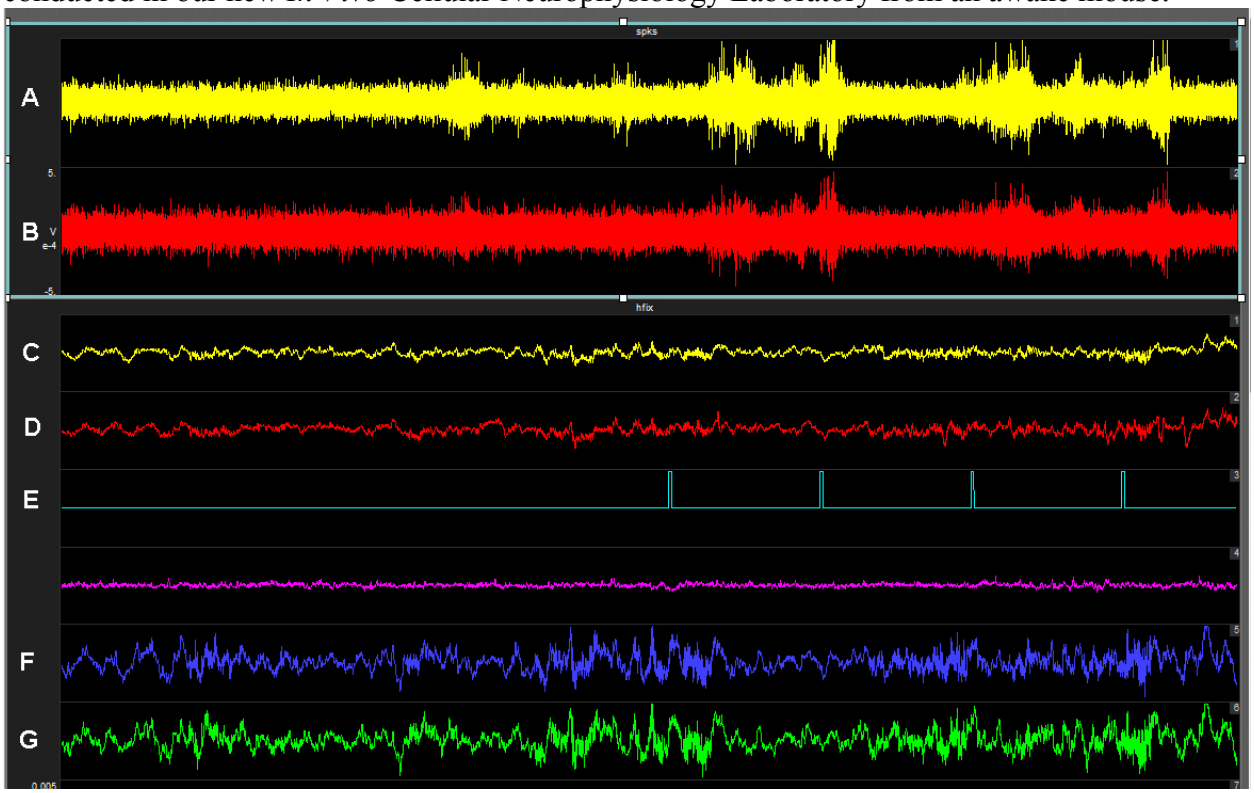

Figure 20. Screenshot of a 60 sec recording illustrating electrophysiological data acquisition using the TDT system. **A, B**: Cortical extracellular activity (MUA) from two different electrodes, **C,D**: EEG recordings from left parietal and right parietal electrodes, **E**: External stimulus signal, **F,G**: LFP traces recorded with the same two electrodes as in A and B.

In March, 2012, we received one breeding pair of *Orexin-tTA; Tet-O ChR2(C128S)* from our collaborator in Japan, Dr. Akihiro Yamanaka. Unfortunately, these mice were infected with *Helicobacter* spp. and thus required an extensive quarantine period upon arrival and they did not breed for more than two months after arrival at SRI International. Once litters were finally produced, we implemented a "cross fostering" protocol to eliminate the *Helicobacter* spp. from newborn pups. On 7/25/12, we received confirmation that some of the offspring are free of *Helicobacter* and can now join the mouse colony room to establish this transgenic line at SRI.

Plans for Year 4:

Task 2a: Analysis of the spatial reference memory study will be submitted for publication.

Task 2b: Data collection for the spatial working memory study will be completed. All data analysis will be completed and the results will be submitted for publication.

Task 2c: We anticipate we will complete the data collection and analysis for the rPVT study.

Task 3a: We will perform immunohistology on the processed tissue to quantify activation of wake-active cell populations defined by immunoreactivity for adenosine deaminase, serotonin, dopamine beta-hydroxylase, and choline acetyltransferase.

Task 3b: We will complete the remaining two lesion studies in Aim 3b, one of which (DBH-saporin lesions of the locus coeruleus) is currently in progress. Analysis of the basal forebrain lesion study will be submitted for publication, and data will be presented at the 2012 Society for Neuroscience meeting in New Orleans.

Task 3c: We anticipate receipt of the first of three transgenic mouse lines (Pet1-Lmx1b(ffp)) within the next several weeks, and we will initiate a breeding program to obtain an age-matched experimental cohort to be ready for study in early 2013. We are also expecting receipt of full cohorts of the remaining two strains (DBH-KO and Hdc-KO) this year, to be studied immediately upon arrival.

Task 4a: We will perform immunohistology on the processed tissue to quantify activation of sleep-active neurons in the ventro-lateral preoptic nucleus. Results on sleep-active cortical nNOS neurons will be presented at the 2012 Society for Neuroscience meeting in New Orleans.

Task 4b: In Year 4, we will continue collecting data from additional animals in order to reach our proposed statistical power of 8 rats per treatment group. The behavioral and neurotransmitter analyses will then be submitted for publication.

Task 6: Having now established the new laboratory and with a "clean" colony of *Orexin-tTA; Tet-O ChR2(C128S)* mice expected to be produced in Year 4, we will conduct Task 6a "Determine whether activation of the Hcrt system is sufficient to induce arousal in the presence of ALM vs. ZOL" as described in the revised Statement of Work.

KEY RESEARCH ACCOMPLISHMENTS

- Spatial reference memory study completed (Figures 1-4).

- Spatial working memory study under undisturbed conditions has been completed. All animals needed to complete the spatial working memory study under sleep-deprived conditions have been implanted and are currently under study.

- Equipment for the rPVT study (Task 2c) has been purchased.

- Publication of "Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone" in *PLoS One* by Morairty et al.

- Completed rat perfusion and tissue processing for histological Tasks 3a and 4a.

- Completed the first of three lesion studies in Aim 3b, evaluating the efficacy of ALM vs. ZOL in basal-forebrain-lesioned or sham-operated animals (Figs 6-7).

- Successfully piloted a locus coeruleus lesion protocol for the second lesion study in Aim 3b (Fig. 8).

- Completed immunohistological analysis of neuronal activation of wake-active hypocretin neurons (Task 3a) and sleep-active cortical nNOS neurons (Task 4a).

- Presented poster entitled "The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake" at the Society for Neuroscience meeting held in Washington, D.C. in 2011 based on data collected in Tasks 2a, 3a and 4a.

- Presented poster entitled "Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat" at the Society for Neuroscience meeting held in Washington, D.C. in 2011 based on data collected in Task 4b.

- Completed the number of animals needed for Task 4b evaluating the effects of oral ALM vs. ZOL on neurotransmitter release in the Sprague-Dawley rat (Figs. 12-16)

- Have preliminary findings of the effects of oral ALM vs. ZOL on animals under conditions of constant wakefulness and recovery sleep (Figs. 17-19)

REPORTABLE OUTCOMES

Vazquez J., A. Nguyen, T. Kilduff. Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat. Program No. 720.09. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

Dittrich L, S. Morairty, A. Wilk, D. Warrier, K. Silveira, T.-M. Chen, T. S. Kilduff. The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake. Program No. 720.10. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

Morairty SR, Revel FG, Malherbe P, Moreau JL, Valladao D, Wettstein JG, Kilduff TS, Borroni E. Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone. *PLoS One* 7(7):e39131. Epub 2012 Jul 2. PMID:22768296.

CONCLUSION

Results continued to accumulate that are consistent with the hypothesis that disfacilitation of wake-promoting systems by the hypocretin (Hcrt) receptor antagonist almorexant (ALM) results in less functional impairment than the inhibition of neural activity produced by the benzodiazepine receptor agonist zolpidem (ZOL). Preclinical data indicate that animals treated with ALM are easily aroused from sleep and are free of ataxia and other behavioral impairments. Measures of both spatial reference memory (Task 2a) and spatial working memory (Task 2b) in

rodents treated with ALM were mostly indistinguishable from vehicle whereas impairments were clearly evident under ZOL. If this observation is confirmed in humans, it would have enormous implications for the management of disturbed sleep in both military and civilian populations. Similarly, wake-active Hcrt neurons can be recruited in the presence of ALM after sleep deprivation but not in the presence of ZOL (Task 3a). Conversely, although both drugs activate sleep-active cortical neurons, sleep-active cells are more strongly activated by ZOL (Task 4a). Lesions of the basal forebrain (BF), a wake-promoting area, potentiated the hypnotic effect of ZOL without affecting the response to ALM (Task 3b), indicating different neural pathways underlie the actions of these two drugs. ALM promoted adenosine and glutamate release in the BF (Task 4b) whereas ZOL promoted GABA release, particularly during waking. An *In Vivo* Cellular Neurophysiology Laboratory with the capabilities to perform the electrophysiology and optogenetic experiments (Tasks 6a-c) was established.

REFERENCES

Dugovic C, Shelton JE, Aluisio LE, Fraser IC, Jiang X, et al. (2009) Blockade of orexin-1 receptors attenuates orexin-2 receptor antagonism-induced sleep promotion in the rat. *J Pharmacol Exp Ther.* 330(1):142-51.

APPENDICES

1. Vazquez J., A. Nguyen, T. Kilduff. Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat. Program No. 720.09. *2011 Neuroscience Meeting Planner*. Washington, DC: Society for Neuroscience, 2011. Online.

2. Dittrich L, S. Morairty, A. Wilk, D. Warrier, K. Silveira, T.-M. Chen, T. S. Kilduff. The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake. Program No. 720.10. *2011 Neuroscience Meeting Planner*. Washington, DC: Society for Neuroscience, 2011. Online.

3. Morairty SR, Revel FG, Malherbe P, Moreau JL, Valladao D, Wettstein JG, Kilduff TS, Borroni E. Dual hypocretin receptor antagonism is more effective for sleep promotion than antagonism of either receptor alone. *PLoS One* 7(7):e39131. Epub 2012 Jul 2. PMID:22768296.

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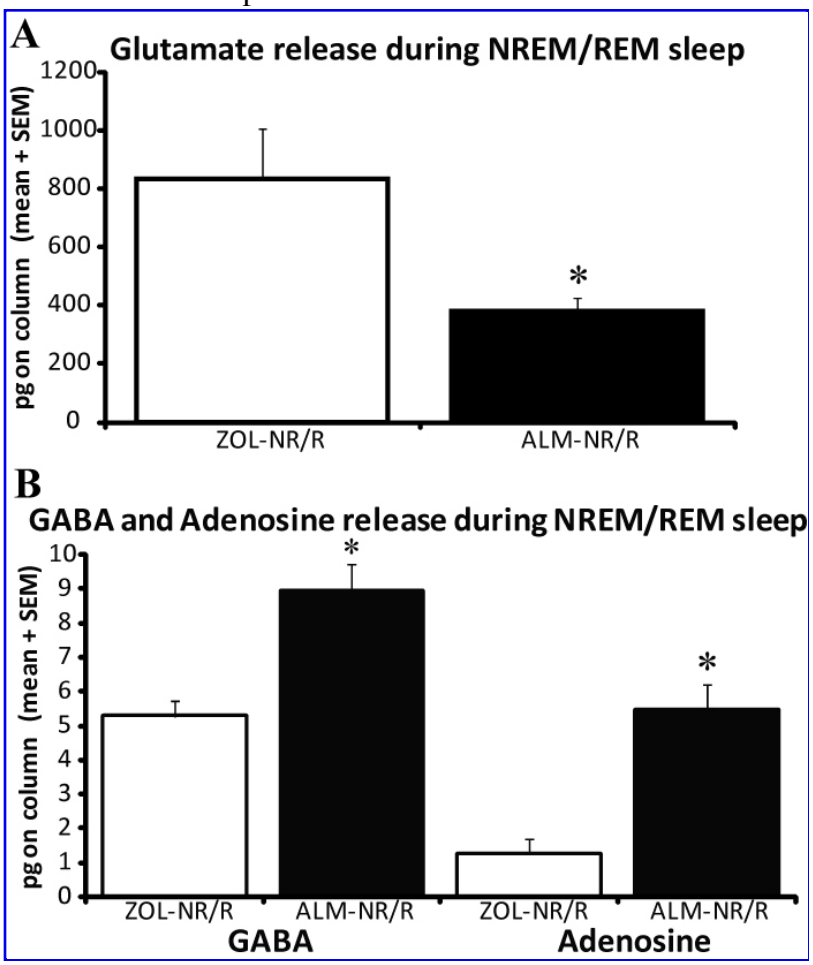
Presentation Abstract

Program#/Poster#:	720.09/XX21
Presentation Title:	Effects of zolpidem and almorexant on basal forebrain neurotransmitter release in freely-moving rat.
Location:	Hall A-C
Presentation time:	Tuesday, Nov 15, 2011, 1:00 PM - 2:00 PM
Authors:	***J. VAZQUEZ**, A. NGUYEN, T. KILDUFF; Ctr. for Neuroscience, Biosci. Div., SRI Intl., MENLO PARK, CA
Abstract:	Hypocretins (orexins) modulate diverse physiological processes such as cognitive function and alertness. Hypocretin-1 and hypocretin-2 (Hcrt) peptides regulate sleep and alertness (Kilduff and Peyron 2000) and Hcrt neurons project to several brain regions including the basal forebrain (BF; Peyron et al. 1998), a brain region critical for promoting wakefulness (Jones 2004). The BF contains cholinergic, GABAergic, and putative glutamatergic neurons important for cortical activation (Manns et al. 2003). Zolpidem (ZOL), a benzodiazepine receptor agonist, affects a Cl^- channel on the $GABA_A$ receptor, resulting in hyperpolarization and somnolence (Dang et al. 2010). In contrast, almorexant (ALM) is a dual Hcrt receptor antagonist that reversibly blocks signaling of both Hcrt peptides. Oral delivery of ALM elicits somnolence without cataplexy and, in rat, decreases active wake and increases the time spent in non-rapid eye movement (NREM) and (REM) sleep (Brisbare-Roch et al. 2007). We tested the hypothesis that oral ALM induces sleep by facilitating the mechanisms that underlie the transition to normal sleep. In contrast to ZOL, which affects $GABA_A$ receptors that are widely distributed in the CNS, ALM acts through blockade of post-synaptic Hcrt receptors, thereby disfacilitating excitation in the BF. We used in vivo microdialysis and HPLC analyses to examine BF glutamate, GABA, and adenosine efflux following oral ZOL (10mg/kg), ALM (100mg/kg), or placebo (VEH) combined with behavioral analyses. Two-way ANOVA revealed a significant drug x state interaction for all neurotransmitters.

Post-hoc comparisons showed that ALM (n=4 rats; $p<0.05$) caused a significant decrease in BF glutamate (**A**) during NREM/REM cycling and the corresponding collection timeframes compared to ZOL (n=4) or VEH (n=3; data not shown). Oral ALM concurrently increased BF GABA and adenosine (**B**; $p<0.05$) during NREM/REM compared to ZOL or VEH. These results provide novel evidence for dynamic neurochemical changes underlying Hcrt modulation of sleep-wakefulness.



Disclosures: **J. Vazquez:** None. **A. Nguyen:** None. **T. Kilduff:** None.

Keyword(s): MICRODIALYSIS

GABA

HYPOCRETIN

Support: USAMRMC W81XWH-09-2-0081

[Authors]. [Abstract Title]. Program No. XXX.XX. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

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Presentation Abstract

Program#/Poster#: 720.10/XX22

Presentation Title: The hypocretin receptor antagonist almorexant induces sleep in rats but does not impair spatial reference memory performance during wake

Location: Hall A-C

Presentation time: Tuesday, Nov 15, 2011, 2:00 PM - 3:00 PM

Authors: ***L. DITTRICH**, S. MORAIRTY, A. WILK, D. WARRIER, K. SILVEIRA, T.-M. CHEN, T. S. KILDUFF;
Biosci., SRI Intl., Menlo Park, CA

Abstract: Most commonly prescribed hypnotics, such as benzodiazepine receptor agonists, cause general inhibition of neural activity. As a result, these hypnotics are less than optimal to aid sleep if there is a risk of being awakened with the need to perform without impairment, e.g., healthcare workers or emergency response personnel. A more specific mechanism of action is exerted by almorexant (ALM), a dual antagonist for hypocretin/orexin (Hcrt) receptors. We hypothesized that challenged rats would be able to stay awake more easily and function with less impairment after a sleep-promoting dose of ALM than after a dose of the benzodiazepine receptor agonist zolpidem (ZOL). To test this hypothesis, we trained 24 rats to remember the location of a platform in a spatial reference memory task (Morris Water Maze). Next day, they were dosed with either ALM (100 mg/kg i.p.), ZOL (30 mg/kg i.p.), or vehicle. Although both drugs induced sleep, the performance of rats dosed with ALM was indistinguishable from the rats dosed with vehicle whereas the group dosed with ZOL displayed weaker preference for the learned location of the platform. To assess the influence of the two compounds on the activity of sleep/wake regulatory neurons, we performed an immunohistological study using c-Fos as a marker of neuronal activity. The same rats were administered the drugs as described above but half of the animals were allowed to sleep for 1.5h after dosing, whereas the remaining rats were sleep deprived by gentle handling. In agreement with the behavioral results, we found that the percentage of

Fos-positive neurons in the wake-active Hcrt neurons in the lateral hypothalamus was higher for sleep deprived animals than for non-sleep deprived animals in the ALM and vehicle groups, whereas there was no such difference for the ZOL group. The sleep-active cortical neurons immunoreactive for neuronal nitric oxide synthase expressed more Fos in animals that were allowed to sleep than in those kept awake, independent from the compound administered. Taken together, our results indicate that ALM effectively induces sleep but unlike ZOL allows the rats to activate wake-promoting neurons and perform normally when needed.

Disclosures: **L. Dittrich:** None. **S. Morairty:** None. **A. Wilk:** None. **D. warrier:** None. **K. Silveira:** None. **T. chen:** None. **T.S. Kilduff:** None.

Keyword(s): WATER MAZE

C-FOS

SLEEP DEPRIVATION

Support: USAMRMC Grant W81XWH-09-2-0081

[Authors]. [Abstract Title]. Program No. XXX.XX. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

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PLoS one

Dual Hypocretin Receptor Antagonism Is More Effective for Sleep Promotion than Antagonism of Either Receptor Alone

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1 Center for Neuroscience and Metabolic Disease Research, SRI International, Menlo Park, California, United States of America, **2** Neuroscience Research, F. Hoffmann-La Roche Ltd., Basel, Switzerland

Abstract

The hypocretin (orexin) system is involved in sleep/wake regulation, and antagonists of both hypocretin receptor type 1 (HCRTR1) and/or HCRTR2 are considered to be potential hypnotic medications. It is currently unclear whether blockade of either or both receptors is more effective for promoting sleep with minimal side effects. Accordingly, we compared the properties of selective HCRTR1 (SB-408124 and SB-334867) and HCRTR2 (EMPA) antagonists with that of the dual HCRTR1/R2 antagonist almorexant in the rat. All 4 antagonists bound to their respective receptors with high affinity and selectivity *in vitro*. Since *in vivo* pharmacokinetic experiments revealed poor brain penetration for SB-408124, SB-334867 was selected for subsequent *in vivo* studies. When injected in the mid-active phase, SB-334867 produced small increases in rapid-eye-movement (REM) and non-REM (NR) sleep. EMPA produced a significant increase in NR only at the highest dose studied. In contrast, almorexant decreased NR latency and increased both NR and REM proportionally throughout the subsequent 6 h without rebound wakefulness. The increased NR was due to a greater number of NR bouts; NR bout duration was unchanged. At the highest dose tested (100 mg/kg), almorexant fragmented sleep architecture by increasing the number of waking and REM bouts. No evidence of cataplexy was observed. HCRTR1 occupancy by almorexant declined 4–6 h post-administration while HCRTR2 occupancy was still elevated after 12 h, revealing a complex relationship between occupancy of HCRT receptors and sleep promotion. We conclude that dual HCRTR1/R2 blockade is more effective in promoting sleep than blockade of either HCRTR alone. In contrast to GABA receptor agonists which induce sleep by generalized inhibition, HCRTR antagonists seem to facilitate sleep by reducing waking "drive".

Citation: Morairty SR, Revel FG, Malherbe P, Moreau J-L, Valladao D, et al. (2012) Dual Hypocretin Receptor Antagonism Is More Effective for Sleep Promotion than Antagonism of Either Receptor Alone. PLoS ONE 7(7): e39131. doi:10.1371/journal.pone.0039131

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Competing Interests: The authors have the following conflicts: Dr. Revel, Dr. Malherbe, Dr. Moreau, Dr. Wettstein, and Dr. Borroni are employed by Roche. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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[9] These authors contributed equally to this work.

Introduction

Determination of the functions of neurotransmitters, neuromodulators and their receptors has classically been aided by use of small molecule receptor-specific antagonists. In recent years, forward and reverse genetics have provided insights into the functions of neurotransmitter/neuromodulatory systems before receptor-specific antagonists were developed. Such was the case for hypocretin (orexin), whose cell bodies in the perifornical and lateral hypothalamus synthesize a pair of neuropeptides alternatively called hypocretin-1 (HCRT1) or orexin-A and hypocretin-2 (HCRT2) or orexin-B [1,2]. Identification of a mutation in the gene encoding HCRT receptor 2 (HCRTR2 or OX2R) as the cause of canine narcolepsy [3] and demonstration that HCRT ligand-deficient mice exhibited periods of behavioral arrest that resembled both human and canine narcolepsy [4] implicated the HCRT system in sleep/wake control well before the first small molecule HCRT receptor antagonists [5,6,7] were described. An extensive literature has since led to the conclusion that the HCRT system is wake-promoting [8,9,10,11] and involved in energy homeostasis [12,13]. Other studies have suggested roles for the HCRT system in neuroendocrine, cardiovascular, water balance, and gastrointestinal control [14], nociception and hyperalgesia [15,16,17], stress and stress-induced analgesia [18,19], reward and addiction [20,21,22,23], and panic anxiety [24].

It is currently unclear whether targeting the HCRTR2 alone or both HCRT receptors is the best strategy for the development of sleep-promoting compounds. Several dual HCRTR1/R2 antagonists show significant sleep-promoting properties [25,26,27,28,29,30,31,32]. However, some reports indicate that HCRTR2 blockade alone was sufficient to produce the hypnotic actions of HCRTR antagonism [32,33]. One study compared the efficacy of the selective HCRTR1 antagonist SB-408124 [34], the selective HCRTR2 antagonist JNJ-10397049 [35], and the dual

antagonist almorexant [27] and concluded that HCRTR1 antagonism attenuates the hypnotic actions of HCRTR2 blockade [32]. While data on the affinity and selectivity of these compounds have been published, the absence of information on their pharmacokinetic properties is problematic for interpretation of their *in vivo* effects.

In the present study, we characterize the hypnotic activity of HCRTR antagonists in rats to determine whether selective or dual HCRTR antagonists are more effective for promoting sleep. To ensure a meaningful *in vivo* comparison, we determined the pharmacological and pharmacokinetic profiles in rats of two selective HCRTR1 antagonists, SB-408124 and SB-334867 [36], the selective HCRTR2 antagonist EMPA [37], and the dual HCRTR1/R2 antagonist almorexant. After showing that SB-408124 displays insufficient brain penetration, we used SB-334867 as the HCRTR1 antagonist for all *in vivo* experiments. Lastly, we determined the time course of HCRTR occupancy by almorexant and correlated this with hypnotic efficacy.

Materials and Methods

Drugs

Almorexant (ACT-078573, (2*R*)-2-[(1*S*)-6,7-Dimethoxy-1-[2-(4-trifluoromethyl-phenyl)-ethyl]-3,4-dihydro-1H-isoquinolin-2-yl]-*N*-methyl-2-phenyl-acetamide) [27], EMPA *N*-(Ethyl-2-[(6-methoxy-pyridin-3-yl)-(toluene-2-sulfonyl)-amino]-*N*-pyridin-3-yl-methyl-acetamide) [37], SB-674042 (1-(5-(2-fluoro-phenyl)-2-methyl-thiazol-4-yl)-1-((S)-2-(5-phenyl-(1,3,4)oxadiazol-2-yl-methyl)-pyrrolidin-1-yl)-methanone) [34], and Cp-5 ((*S*)-1-(6,7-Dimethoxy-3,4-dihydro-1H-isoquinolin-2-yl)-3,3-dimethyl-2-[(pyridin-4-ylmethyl)-amino]-butan-1-one) [7] were synthesized at F. Hoffmann-La Roche Ltd. (Basel, Switzerland) or SRI International (Menlo Park, CA USA) according to the patent literature [38]. SB-334867 (1-(2-methylbenzoxazol-6-yl)-3-[1,5]naphthyridin-4-yl-urea hydrochloride), zolpidem (N,N,6-Trimehtyl-2-(methylphenyl)-imidazol[1,2-a]pyridine-3-acetamide) and SB-408124 (1-(6,8-difluoro-2-methyl-quinolin-4-yl)-3-(4-dimethylamino-phenyl)-urea) were purchased from Tocris Bioscience (Ellisville, MO). Chemical structures are provided in Figure S1. [^{3}H]almorexant (specific activity: 42.7 Ci/mmol), [^{3}H]SB-674042 (specific activity: 24.4 Ci/mmol) and [^{3}H]EMPA (specific activity: 94.3 Ci/mmol) were synthesized at Roche.

Animals

Animal experiments performed at F. Hoffmann-La Roche were conducted in strict adherence to the Swiss federal regulations on animal protection and to the rules of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), and with the explicit approval of the local Cantonal Veterinary Office/Authority Basel City. Animal experiments performed at SRI International were approved by SRI's Institutional Animal Care and Use Committee and were in accordance with U.S. National Institute of Health guidelines. Male Wistar rats (240±20 g) used for spontaneous locomotion studies and pharmacokinetic studies at F. Hoffmann-La Roche were obtained from RCC Ltd. (Fullinsdorf, Switzerland). Male Sprague-Dawley rats (300±25 g) used for receptor occupancy studies at F. Hoffmann-La Roche were from Iffa Credo (Lyon, France). Animals were housed in separate rooms under a 12 h light/12 h dark cycle (light onset: 06:00, except where noted below; Zeitgeber time 0, ZT0) at 22±2°C, with *ad libitum* access to food and water. Male Sprague-Dawley rats (300±25 g) used for sleep studies at SRI were from Charles River (Wilmington, MA) and were housed in a temperature-controlled recording room under a 12 h light/12 h dark cycle (lights on at 05:00) with food and water available *ad libitum*. Room temperature (24±2°C), humidity (50±20% relative humidity), and lighting conditions were monitored continuously via computer. Animals were inspected daily in accordance with AAALAC and SRI guidelines.

Pharmacological Studies

[^{3}H]almorexant binding to rat HCRTR1 and HCRTR2. The rat cDNAs encoding HCRTR1 (Accession No. P56718) and HCRTR2 (Accession No. P56719) were subcloned into pCI-Neo expression vectors (Promega, Madison, WI) and used to transfect HEK293 cells (acquired commercially from ATCC-LGC, Molsheim, France) as previously described [37]. Membrane preparations, saturation and inhibition experiments, and determination of the association and dissociation kinetic parameters of [^{3}H]almorexant to rHCRTR2-HEK293 cell membranes were performed at F. Hoffmann-La Roche as previously described [37] and reported in the Materials and Methods S1.

Pharmacokinetic Studies

Pharmacokinetic analyses were performed at F. Hoffmann-La Roche as described in supporting Maqterials and Methods S1.

SB-334867 selectivity screen. SB-334867 was evaluated in a selectivity screen performed at CEREP (Paris, France). The screen consisted of binding assays on a panel of 79 target receptors. The specific binding (SB) of a radioligand to each target receptor was defined as the difference between the total binding and the nonspecific binding determined in the presence of a cold competitor in excess. The results are expressed as a percent of control SB obtained in the presence of SB-334867 used at 10 μM. Details on the CEREP screen are available from www.cerep.fr.

Effect of Almorexant and SB-334867 on Spontaneous Locomotor Activity in Rats

Locomotor activity (LMA) was evaluated at F. Hoffmann-La Roche as described previously [39]. Male Wistar rats were placed for 2 weeks in a 12 h light/12 h dark cycle with light onset at 22:00 (ZT0). Three h after the onset of the dark period (i.e., ZT15), rats were injected ip with either vehicle or HCRT receptor antagonist (almorexant or SB-334867 at 3, 10, 30 mg/kg in NaCl 0.9%, 0.3% Tween-80) (n = 8 per group), and returned to the recording chambers. Spontaneous LMA was recorded for a period of 30 min. At the end of the experiment, the brain and plasma were collected for determination of the drug exposure and brain/plasma concentration ratio.

Electroencephalogram, Core Body Temperature and Locomotor Activity Studies

Surgical procedures and recordings. All rodent electroencephalograph (EEG) studies were performed at SRI International. Three groups of eight male Sprague-Dawley rats (300±25 g; Charles River, Wilmington, MA) were implanted with chronic recording devices (F40-EET, Data Sciences Inc., St Paul, MN) for continuous recordings of EEG, electromyograph (EMG), core body temperature (T_{core}), and LMA via telemetry as previously described [40]. Data recording and scoring were performed as previously reported [40] (see also Supplemental Material and Methods). The EEG and EMG data were scored in 10 sec epochs for waking (W), rapid eye movement sleep (REM), and non-REM sleep (NR). T_{core} and LMA (counts per minute) were analyzed as hourly means. Data from the EEG studies are

reported in hourly means such that the hourly time ZT1 refers to the hour between time points ZT0 and ZT1.

Experimental design. For each of the three separate studies, a repeated measures counter-balanced design was employed in which each rat received five separate dosings. The dosing conditions for study 1 included SB-334867 at three concentrations (3, 10 and 30 mg/kg), zolpidem (ZOL, 7.5 mg/kg) and a vehicle control (saline 95%/ethanol 5%). The dosing conditions for study 2 included EMPA at three concentrations (10, 30 and100 mg/kg), ZOL (10 mg/kg) and a vehicle control (HPMC). The dosing conditions for study 3 included almorexant at three concentrations (10, 30 and 100 mg/kg), ZOL (10 mg/kg) and a vehicle control (HPMC). All dosings were administered ip in a volume of 2 ml/kg. A minimum of 3 d elapsed between doses. Dosing occurred during the middle of the rats' normal active period at the start of ZT19 and was typically completed within 10 min. Animals were continuously recorded for 6 h prior to dosing and for 18 h following dosing.

Determination of HCRTR1 and HCRTR2 occupancy by almorexant. This study was performed at F. Hoffmann-La Roche. Sixty-five male Sprague-Dawley rats, housed 5 per cage (light onset: 12:00), were injected intraperitoneally (ip) with either vehicle (1.25% hydroxypropyl methylcellulose (HPMC), 0.1% docusate sodium) or almorexant (30 mg/kg in 1.25% HPMC, 0.1% docusate sodium) at the mid-dark phase (ZT18; i.e., 6 h after lights-off), and returned to their home cage. Groups (n = 5 per group) of vehicle- or almorexant-treated animals were then sacrificed by decapitation 0.5, 2, 4, 8 or 12 h after the injection. An extra group of non-injected rats (n = 5) was also sampled at ZT18. Plasma was collected and stored at −80°C until assayed. Brains were rapidly dissected, frozen on dry ice, and stored at −80°C. Series of coronal brain sections (14 µm) were cut in a cryostat through the posterior hypothalamus (tuberomammillary nucleus level: 3.8 to 4.2 mm posterior to bregma) and the brain stem (dorsal raphe nucleus level: 7.3 to 8 mm posterior to bregma; locus coeruleus level: −9.3 to −10 mm posterior to bregma), thaw-mounted (6 sections per slide) and stored at −20°C. After sectioning, the remaining pieces of brain were kept at −80°C for later determination of almorexant brain concentration. The brain and plasma concentrations of almorexant were determined by quantitative liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS).

Receptor occupancy (RO) was determined as published previously [41]. For each Hcrt receptor subtype, two series of slides were thawed and incubated at room temperature with the relevant radioligand in assay buffer for 15 min (HCRTR1) or 1 h (HCRTR2). For HCRTR1, assay buffer (2 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 100 µL per section) contained either 5 nM [^{3}H]SB-674042 (for determination of total binding, TB) or 5 nM [^{3}H]SB-674042 plus 10 µM SB-408124 (for determination of non-specific binding, NSB). For HCRTR2, assay buffer (1 mM $CaCl_2$, 5 mM $MgCl_2$, 25 mM HEPES, pH 7.4, 120 µL per section) contained either 1 nM [^{3}H]EMPA (for determination of TB) or 1 nM [^{3}H]EMPA plus 10 µM Cp-5 (for determination of NSB). The liquid was drained, the brain sections were rinsed with ice-cold assay buffer (2 brief washes followed by 3×2 min soaking) and distilled water (3 brief dips) and air dried at 4°C for 12 h. The slides were exposed together with [^{3}H] microscales against tritium-sensitive imaging plates (BAS-TR2025) for 5 days. The plates were scanned with a high resolution phosphor imager device (Fujifilm BAS-5000) and calibrated measurements of radioactivity (fmol/mg protein) were made. All analyses were performed blind to treatment.

For each selected region, the mean signal density (TB) was measured and averaged from three consecutive sections from the same slide. The specific binding (SB) signal was then determined for each animal by subtracting the NSB signal from the TB signal. NSB was measured from adjacent brains sections incubated with the radiotracer and an excess of cold competitor. The SB signal was averaged for each experimental group and the percent RO was calculated at each time-point according to the equation $RO = (1-(SB_{almorexant}/SB_{vehicle}))\times 100$, where $SB_{almorexant}$ is the average SB for the animal group injected with almorexant and $SB_{vehicle}$ is the average SB for the animal group injected with vehicle.

Statistical Analyses

Results are shown as mean±SEM. LMA and RO data were analyzed with one-way ANOVA followed by Dunnett's analysis. EEG data were analyzed with repeated measures (rm)-ANOVA, followed by paired two-tailed *t*-tests. REM:NR ratios, sleep latencies (NR and REM) and cumulative data were analyzed with one-way rm-ANOVA and all other data with two-way rm-ANOVA. Light period and dark period data were analyzed separately as well as pre- and post-drug administration data. Statistical significance was set at $P<0.05$.

Results

Pharmacological Studies

Binding characteristics of [^{3}H]almorexant to rHCRTR1- and rHCRTR2-expressing cell membranes. To characterize the *in vitro* binding of [^{3}H]almorexant to rat HCRT receptors, saturation binding analyses were performed at binding equilibrium on membranes isolated from HEK293 cells transiently transfected with rHCRTR1 and rHCRTR2. As shown in Fig. 1A and B, [^{3}H]almorexant bound with high affinity to a single saturable site on recombinant rHCRTR1 (K_d of 3.4±0.3 nM and B_{max} of 27.2±0.7 pmol/mg prot, at 23°C) and rHCRTR2 (K_d of 0.5±0.0 nM and B_{max} of 53.0±1.4 pmol/mg prot, measured at 37°C). Binding kinetics of [^{3}H]almorexant to membrane preparations from HEK293 cells transiently expressing rHCRTR2 are shown in Fig. 1C and D and the kinetic parameters in Table 1. The association binding of [^{3}H]almorexant to the rHCRTR2 had a half-maximal binding at 10 min and reached equilibrium within 50 min. The data were fit to a one-phase exponential model with the association rate constant of 0.073±0.015 $nM^{-1}min^{-1}$. The dissociation rate for [^{3}H]almorexant binding to the rHCRTR2 was determined by the addition of an excess amount of almorexant (5 µM) after equilibrium was reached. The rate of [^{3}H]almorexant dissociation from rHCRTR2 membrane was slow; the reversal of binding was incomplete and did not reach baseline even after 2 h (Fig. 1D & Table 1).

The potencies of almorexant and of the selective HCRTR1 antagonists SB-334867 [6] and SB-408124 [34] in inhibiting [^{3}H]almorexant binding to HEK293-rHCRTR1 and HEK293-rHCRTR2 cell membranes are given in Table 2. Almorexant was able to displace [^{3}H]almorexant binding from rHCRTR1 and rHCRTR2 membranes with high affinity (Table 2). In contrast, SB-334867 and SB-408124 displaced [^{3}H]almorexant binding from rHCRTR1, but not from rHCRTR2, with high affinity (Table 2).

Pharmacokinetic Studies

Pharmacokinetic properties of SB-334867, SB-408124, EMPA and almorexant in rats. The oral bioavailability and pharmacokinetic properties of almorexant, SB-334867 and SB-408124 were evaluated in Wistar rats. The mean pharmacokinetic parameters after single iv or oral (po) bolus administration in rat

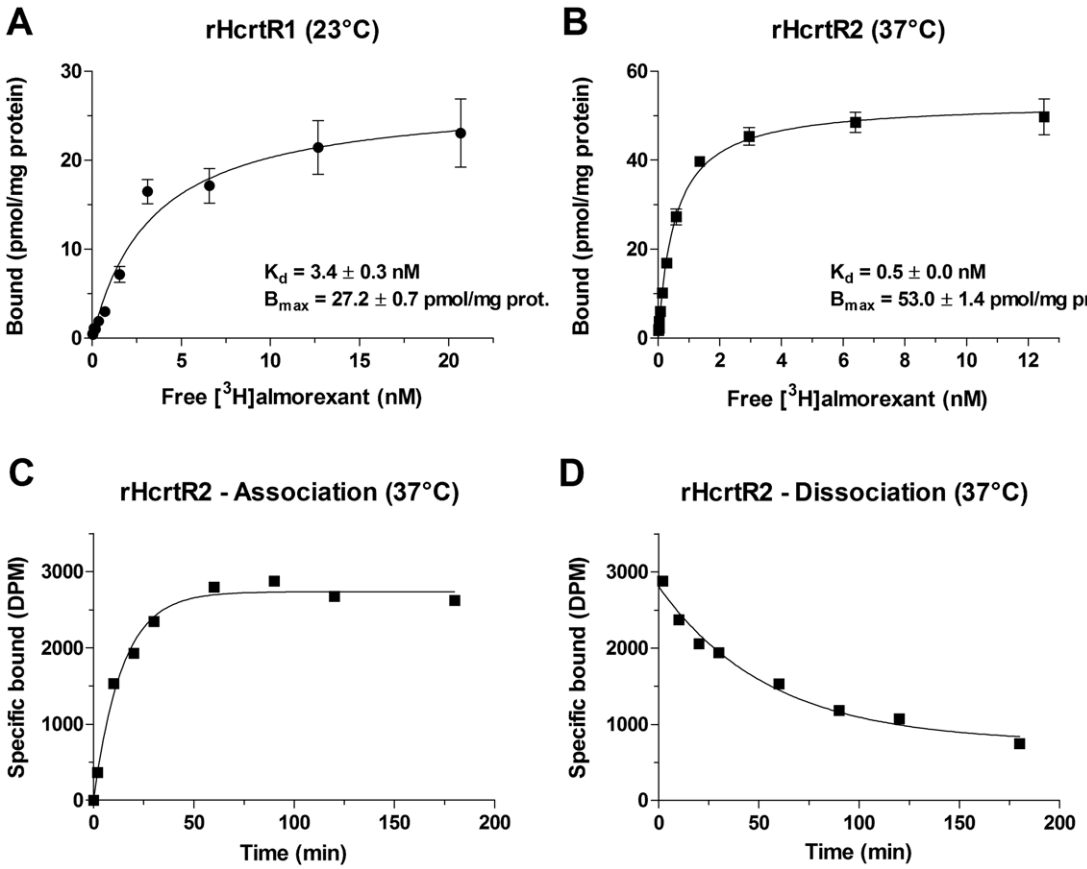

Figure 1. Binding characteristics of [^{3}H]almorexant to rHCRTR1 and rHCRTR2 cell membranes. (**A,B**) Saturation binding curves of [^{3}H]almorexant binding to membranes from HEK293 cells transiently transfected with rHCRTR1 (**A**) or rHCRTR2 (**B**). Each data point represents the mean±SEM of three independent experiments performed in triplicate. The data were analyzed by nonlinear regression analysis using GraphPad Prism 4.0 software and a single-site binding model. (**C,D**) Time course for the association (**C**) and dissociation (**D**) of [^{3}H]almorexant binding to rHCRTR2 membranes.
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are given in Table S1. Almorexant displayed a high systemic plasma clearance, high volume of distribution at steady state (Vss) and low oral bioavailability in rat. In addition, almorexant was highly bound to plasma proteins (<3.7%, and <8.7% free fraction in human and rat plasma, respectively), and its stability measured for 2 h in human and rat plasma was 90.0% and 95.0%, respectively. The mean brain/plasma concentration ratio of almorexant was 0.12 in rat.

SB-334867 exhibited a low systemic plasma clearance, medium Vss and oral bioavailability in rat. SB-334867 is highly bound to plasma proteins (1.3%, and 0.8% free fraction in human and rat plasma, respectively), and its stability measured for 1 h/4 h in human and rat plasma was 95%/93% and 104%/110%, respectively. The mean brain/plasma concentration ratio of SB-334867 (at a dose of 8.8 mg/kg, po) was 0.53 in rat.

SB-408124 had a low systemic plasma clearance, low Vss and medium oral bioavailability in rat. SB-408124 had very low free fraction in human and rat (0.3% and <0.1%, respectively) and its stability (1 h/4 h) in human and rat plasma was 94%/88% and 101%/107%, respectively. The mean brain/plasma concentration ratio of SB-408124 (at dose of 18 mg/kg, po) was 0.03 in rat. Such unfavorable pharmacokinetic properties of SB-408124, most importantly its extremely low brain penetration, prompted us to use SB-334867 for further *in vivo* studies in the rat.

The pharmacokinetic profiles of EMPA have been reported previously [37].

Table 1. Kinetic parameters for the association and dissociation of [^{3}H]almorexant in rHCRTR2-HEK293 cell membranes at 37°C.

Compound	Association kinetic	Dissociation kinetic	Apparent	
	K_{on} ($nM^{-1}min^{-1}$)	K_{off} (min^{-1})	$t_{1/2}$ (min)	K_d (nM)
[^{3}H]almorexant	0.073±0.015	0.021±0.004	36.3±5.7	0.33±0.9

The K_{on} (calculated on rate), K_{off} (observed off rate), $t_{1/2}$ (half-maximal binding) and K_d (apparent dissociation constant) values are ± SEM, calculated from three independent experiments (each performed in quadruplicate) as described under ''Materials and Methods''.
doi:10.1371/journal.pone.0039131.t001

Table 2. Potencies of almorexant, SB-408124 and SB-334867 antagonists in inhibition of [^{3}H]almorexant binding to the membrane preparations from HEK293 cells transiently expressing rHCRTR1 and rHCRTR2.

Compound	rHCRTR1	rHCRTR2
	[^{3}H]almorexant (23°C)	[^{3}H]almorexant (37°C)
	K_i (nM)	K_i (nM)
almorexant	7.1±0.7	2.0±0.0
SB-408124	45.7±4.1	5370.0±2200.0
SB-334867	58.4±2.9	2390.0±81.0

[^{3}H]almorexant was used at a concentration equal to its K_d values of 3.4 nM and 0.5 nM at rHcrtR1 and rHcrtR2, respectively, in these competition binding experiments. K_i values for [^{3}H]almorexant binding inhibition by various antagonists were calculated as described under "Materials and Methods". Values are ± SEM of the K_i calculated from three independent experiments, each performed in duplicate.

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Selectivity profile of SB-334867. The specificity of SB-334867 at the HCRTR1 was confirmed by assessment in radioligand binding assays in a broad CEREP screen (Paris, France; www.cerep.fr) (Table S2). Among the 79 receptors tested, 30 were peptide receptors. SB-334867 was inactive (<40% activity at 10 μM) at all targets tested with the exception of the A_{2A} (adenosine), A_3, MT3 (melatonin), P_{2Y} (purinergic 2Y) and $5HT_{2C}$ (serotonin 2C) receptors, where it caused 89%, 63%, 102%, 64% and 70% displacement of specific binding at 10 μM, respectively. The selectivity profiles of almorexant [27] and EMPA [37] have been reported previously.

Effect of Almorexant and SB-334867 on Spontaneous Locomotor Activity in Rats

The ability of almorexant and SB-334867 to antagonize *in vivo* the biological action of endogenous hypocretins was assessed by measuring spontaneous LMA during the active phase. Almorexant dose-dependently reduced LMA, although only the 30 mg/kg dose reached significance when compared to vehicle (Figure 2A; $F = 4.28$, $p<0.05$). Similarly, SB-334867 dose-dependently reduced spontaneous LMA, with both the 10 and 30 mg/kg doses being statistically different from vehicle (Figure 2B; vehicle: 6097±536; 10 mg/kg: 3509±383; 30 mg/kg: 2626±341; $F = 12.80$, $p<0.01$ and $p<0.001$, respectively).

The plasma and brain exposure of SB-334867 were measured at the end of the LMA experiment. When determined 35 min after ip administration, SB-334867 doses of 3, 10 and 30 mg/kg produced plasma levels of 220, 718 and 738 ng/mL vs. brain levels of 48, 171, and 142 ng/mL (ratios: 0.21, 0.23, 0.19, respectively). These results confirmed the ability of SB-334867 to enter the rat brain at the doses used in this report.

Rodent EEG Studies

The effects of almorexant, SB-334867 and EMPA administered in the middle of the dark (active) period were evaluated during the latter half of the active period and subsequent light (inactive) period to determine both efficacy for sleep promotion and whether "hangover" or rebound effects occurred. Of these three compounds, only almorexant reduced NR and REM sleep latency (Figure 3). Almorexant at 30 and 100 mg/kg reduced NR latency while only the 30 mg/kg concentration decreased latency to REM sleep. ZOL produced a decrease in NR latency in all three experiments.

In contrast, all three compounds increased NR sleep (Figure 4). SB-334867 at 3 and 30 mg/kg increased cumulative NR for the first 4 and 6 h periods following administration ($F = 10.808$, $p<0.0001$ and $F = 10.752$, $p<0.0001$, respectively). EMPA at 100 mg/kg also increased cumulative NR for the first 4 and 6 h periods post administration ($F = 17.655$, $p<0.0001$ and $F = 12.816$, $p<0.0001$, respectively). Almorexant had the strongest effect: both 30 and 100 mg/kg increased cumulative NR for 2, 4 and 6 h following administration ($F = 13.010$, $p<0.0001$; $F = 17.771$, $p<0.0001$; and $F = 16.179$, $p<0.0001$, respectively). Cumulative REM also increased for the first 2 h following almorexant at 30 mg/kg ($F = 5.418$, $p = 0.0023$) and for the 6 h period following the 100 mg/kg dose (Figure 4; $F = 8.535$, $p<0.0001$). ZOL increased cumulative NR and decreased cumulative REM in all three experiments. Whereas ZOL suppressed the REM:NR ratio in all 3 studies, none of the 3 test compounds did (Table 3). Although ZOL had significant effects on EEG delta power during NR, this parameter was little affected by any of the three test compounds compared to vehicle control (Figure S2).

There were few effects on sleep/wake amounts during the light period subsequent to administration of EMPA, SB-334867 or almorexant (Figure 5). REM was not significantly affected during this period following any of the three HCRT antagonists. NR decreased during the third hour of the light period (ZT3) following SB-334867 at 10 and 30 mg/kg while NR increased during ZT1 and ZT6 following almorexant at 30 mg/kg compared to vehicle. No significant effects on NR were found following EMPA during the light period.

Significant results occurred in measures of sleep-wake consolidation (Tables S3, S4, S5 and Figures S3, S4, S5). The strongest effects were found following almorexant at 100 mg/kg, which produced increased numbers of W and NR bouts during ZT19, ZT20, and ZT22-ZT24 ($F = 2.069$, $p = 0.0077$ and $F = 2.413$, $P = 0.0015$, respectively). The number of REM bouts was increased by almorexant at 100 mg/kg during ZT22-ZT24 ($F = 2.963$, $p = 0.002$). W bout duration was decreased following almorexant at 100 mg/kg during ZT22 compared to vehicle ($F = 2.320$, $p = 0.0023$). All three concentrations of EMPA increased the number of W bouts ($F = 4.243$, $p = 0.0065$). SB-334867 increased NR bout duration during ZT21 following 30 mg/kg and during ZT24 following 3 mg/kg ($F = 4.574$, $p<0.0001$).

Both LMA and T_{core} underwent dose-dependent decreases after drug treatment (Figure 6). ANOVA revealed condition effects for both almorexant and EMPA in which LMA was decreased across the 6 h period following administration of both compounds at 100 mg/kg compared to vehicle ($F = 7.316$, $p<0.00015$ and $F = 7.442$, $p = 0.00018$ respectively). No differences in LMA during the subsequent light period were found. Condition effects for T_{core} were found in all three studies. The high concentrations tested for all three HCRT receptor antagonists decreased T_{core} across the 6 h period following administration ($F = 7.629$, $p = 0.00027$ for SB-334867; $F = 7.442$, $p = 0.00018$ for EMPA; $F = 7.315$, $p = 0.00036$ for almorexant). ZOL administration resulted in the largest declines in T_{core} in all three studies, which was followed by a sustained rebound increase in T_{core} during the subsequent light period.

Time Course of HCRT Receptor Occupancy (RO) by Almorexant

To determine the time-course of HCRTR1 and HCRTR2 RO by almorexant, a single dose of almorexant at the smallest concentration shown to promote sleep (30 mg/kg, ip; Figure 4) was administered in the mid-dark phase (ZT18) and rats were

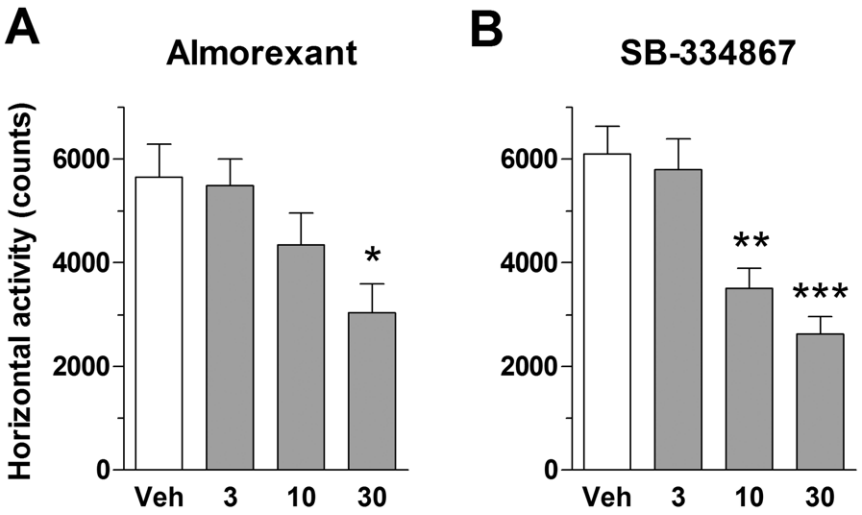

Figure 2. Effects of almorexant and SB-334867 on spontaneous locomotor activity of rats during the active phase. Both almorexant **(A)** and SB-334867 **(B)** reduced locomotor activity compared to vehicle (Veh) when administered 3 h after the onset of the dark period. Horizontal locomotor activity was recorded for a period of 30 min. Numbers on the X-axes represent intraperitoneal doses in mg/kg.***$p<0.001$, **$p<0.01$, *$p<0.05$ vs. Veh (one-way ANOVA followed by Dunnett's analysis). All data are mean±SEM (n = 8 per group).
doi:10.1371/journal.pone.0039131.g002

sacrificed after incubation periods of 0, 0.5, 2, 4, 8 or 12 h. For both HCRTR1 and HCRTR2, the NSB was minimal and represented 6.2% and 3%, respectively, of the average TB signal measured in control animals. The signal localization was in good agreement with the distribution of *HCRTR1*- and *HCRTR2*-expressing neurons [42,43], as confirmed by *in situ* hybridization on separate sections (data not shown). Figure 7A shows representative autoradiograms of HCRTR1 binding sites in the locus coeruleus (*LC)*. This signal localization is in good agreement with the distribution of *Hcrtr1*-expressing neurons [42,43], as confirmed by *in situ* hybridization (data not shown). The rats injected with vehicle displayed maximal HCRTR1 radiotracer binding at all time points (Figure 7A), whereas the animals injected with almorexant showed reduced binding 2 h after the injection. Binding of the HCRTR1 radiotracer returned to levels similar to control 8–12 h post almorexant injection.

Figure 7B shows representative autoradiograms of the HCRTR2 binding sites examined at 2 different rostro-caudal levels. At the level of the posterior hypothalamus, signal was observed in various brain regions, including the tuberomammillary nuclei (*TMN*), cerebral cortex (*CC*), retrosplenial cortex (*RSC*), and field CA3 of the hippocampus (*CA3*). The signal attributed to the *TMN* was verified by *in situ* hybridization for histidine decarboxylase mRNA on separate sections (data not shown). At the level of the anterior pons, the dorsal raphe nuclei (*DRN*), pontine nuclei (*Pn*) and parabigeminal nuclei (*PBG*) displayed specific labeling. This pattern corresponds to that already reported by Malherbe et al. [37] and was in good agreement with the distribution of *Hcrtr2*-expressing neurons previously described [42,43]. The rats injected with vehicle displayed constant HCRTR2 binding at all time points. In contrast, the animals that received almorexant exhibited a very strong reduction of HCRTR2 radiotracer binding and, 2 h after almorexant injection, no signal could be detected (Figure 7B). Reduction of TB signal was still evident for all brain regions 12 h after almorexant administration.

SB was quantified in the *LC* for HCRTR1 and in 6 brain areas (*TMN*, *CC*, *CA3*, *RSC*, *DRN* and *Pn*) for HCRTR2, and the RO by almorexant was determined for 12 h post-injection (Figure 7C and Figure S7). HCRTR1 RO reached 50–60% from 30 min to 4 h post-injection (maximum: 59% after 2 h) and then returned to basal levels after 6 h. This RO profile paralleled that of almorexant concentration in the plasma (Figure 7D) and brain (Figure S6). For both compartments, drug concentration rose rapidly and reached a peak around 30 min, with plasma levels of 1966.4±349.2 ng/mL and brain levels of 565.8±112.4 ng/g (mean brain/plasma concentration ratio: 0.28). The half-maximal concentrations were achieved between 4 and 6 h.

For HCRTR2, all 6 structures displayed a comparable RO profile (Figure 7C for *DRN* and *TMN*, and Figure S7 for *CC, RSC, Pn* and *CA3*): it was close to 100% within 30 min after dosing, remained at maximal levels at 2 h and 4 h, and started to slowly decline between 4 and 6 h. After 12 h, although the brain and plasma levels of almorexant were strongly reduced (Figure 7C and Figure S6), HCRTR2 occupancy was still elevated with levels between 49 and 67%, depending on brain structure (Figure 7C and Figure S7; *TMN*: 49.2±13.2%; *CC*: 66.1±11.6%; *CA3*:58.4±11.5%; *RSC*: 64.6±10.7%; *DRN*: 57.7±10.5%; *Pn*: 67.2±13.9%).

Discussion

This study was undertaken to determine whether blockade of either or both HCRT receptors is more effective in promoting sleep. Multiple dual HCRTR1/R2 antagonists employing different molecular scaffolds have been found to have significant significant sleep-promoting properties [25,26,27,28,29,30,31] Anatomical localization of HCRTRs suggests that both receptors are involved in the promotion of wakefulness [39,43]. High levels of HCRTR1 are found in *LC* while only HCRTR2 is abundant in the *TMN*. Both receptors are expressed at moderately high levels in the dorsal and medial raphe and in the cholinergic regions of the basal forebrain. In the laterodorsal tegmentum and the pedunculopontine nucleus (brain stem cholinergic regions), the HCRTR1 is predominant. However, some recent reports support the hypothesis that only blockade of the HCRTR2 underlies the hypnotic actions of HCRTR antagonism [30,31]. Further, one study suggests that antagonism

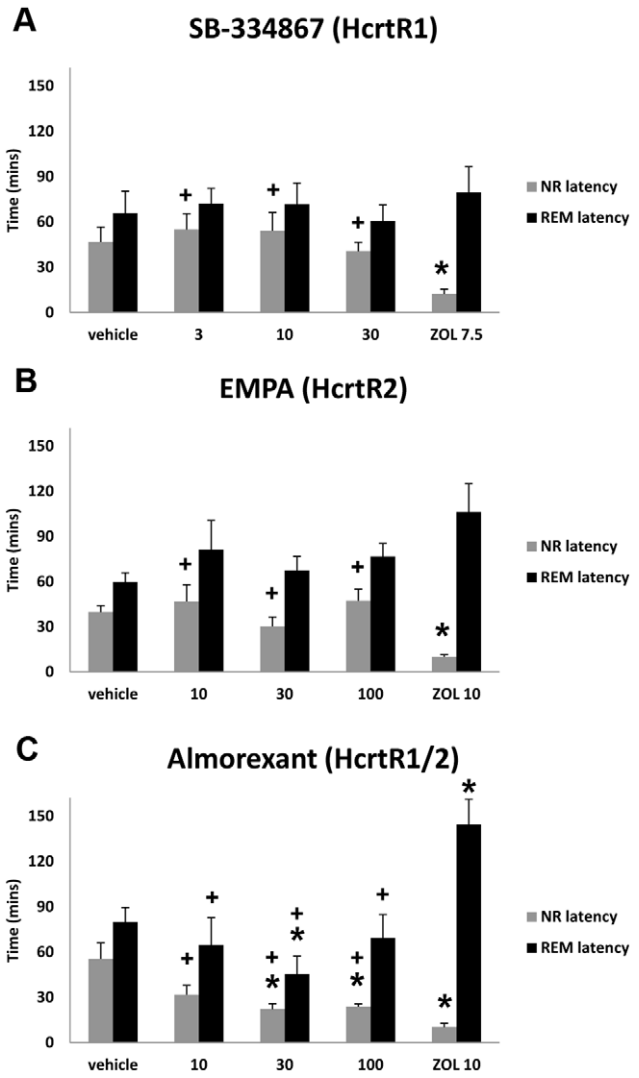

Figure 3. Latency to the onset of NR and REM sleep following administration of SB-334867. (A), EMPA **(B)**, and almorexant **(C)** as compared to zolpidem (ZOL). *=significantly different from vehicle ($p<0.05$); +=significantly different from ZOL ($p<0.05$) (One-way repeated measures ANOVA followed by paired two-tail *t* tests; n=8 per group). Data represent the mean±SEM.
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of HCRTR1 attenuates the hypnotic actions of HCRTR2 blockade [32]. Therefore, to help clarify the hypnotic effects of HCRTR blockade, we characterized the pharmacological and pharmacokinetic properties of selective and dual HCRTR antagonists in rat before evaluating their relative efficacy on sleep and wakefulness.

Pharmacokinetic Considerations

The affinities of almorexant, SB-408124 and SB-334867 at the rat HCRTR1 and HCRTR2 receptors are very similar to those reported for human HCRT receptors (for almorexant, K_i values of 4.7 nM and 0.9 nM at hHCRTR1 and hHCRTR2, 37°C, respectively [42]; for SB-334867, K_i value of 38.7 nM at rHCRTR1 [34]; for SB-408124, K_i value of 26.9 nM at rHCRTR1 [34]). Almorexant had high affinity for both HCRTRs and displayed a slow rate of dissociation from rHCRTR2 membranes *in vitro*, which translated into a long-lasting occupancy of the HCRTR2 *in vivo*. This property likely underlies some of the pharmacological effects described here. Among the three antagonists tested, almorexant had the highest systemic plasma clearance, highest Vss but lowest oral bioavailability; both SB-334867 and SB-408124 had low clearances and medium to low bioavailability. Importantly, SB-408124 had a very low free fraction and was found to penetrate the brain poorly, especially when compared to the other compounds. This prompted us to use SB-334867 for evaluating the effects of selective HCRTR1 blockade on sleep.

Effects of Selective HCRTR1 and HCRTR2 Antagonists on Sleep/wake

Selective blockade of HCRTR2 clearly results in sleep promotion. The HCRTR2 antagonist JNJ-10397049 reduced NR latency during both the light and dark phases, increased NR duration in the light phase, and increased both NR and REM duration during the dark phase [30,31]. Here, although EMPA had no effect on either NR or REM latency when administered in the mid-dark phase, it increased cumulative NR for the first 4 and 6 h. Conversely, icv infusion of an HCRTR2 agonist, [Ala^{11}]orexin-B, during the light period dose-dependently increased wake duration and decreased the amounts of both NR and REM sleep [44]. The effects of HCRT1 (orexin-A) on wakefulness and NREM sleep were reduced more in $OX2R^{-/-}$ mice than in $OX1R^{-/-}$ mice, implying that HCRTR2 has a greater influence than HCRTR1 on these parameters, at least in mice [45].

The selective HCRTR1 antagonist SB-334867 dose-dependently reduced LMA and, at 3 and 30 mg/kg i.p., increased cumulative NR for the first 4 and 6 h. These results differ from those of Dugovic *et al.* [32] who reported that selective blockade of HCRTR1 using SB-408124 had no effect on sleep, although it reduced LMA. However, the time of drug administration differed between these studies (middle vs. start of the active phase). By the middle of the active phase, both endogenous HCRT tone [46,47] and sleep pressure are increased, so HCRTR antagonists are more likely to be effective at this time of day than at dark onset.

A previous study showed that SB-334867 blocked HCRT1-induced effects on REM sleep but did not alter any sleep parameters when administered alone [36]. However, only the first hour after treatment was examined whereas, here, effects of SB-334867 on sleep were only apparent after 2 h. Importantly, we showed that SB-408124 exhibits poor pharmacokinetic properties, with notably low free fraction and little brain penetration, which likely limits its *in vivo* efficacy. The brain-to-plasma ratio for SB-408124 is 0.03, which is in the range of blood contamination levels obtained with the residual blood carried over in the brain homogenate (in the absence of compound in the brain). Although Dugovic *et al.* [32] did not specifically report brain-to-plasma ratios, they did report both brain and plasma concentrations following administration of SB-408124 at 30 mg/kg. Using these numbers, a brain-to-plasma ratio for SB-408124 is calculated to be 0.012 (using C_{max} values given in text: brain-to-plasma ratio = 1.09/84.29 = 0.012), which is in good agreement with our findings. This observation most likely explains why Dugovic *et al.* [32] did not detect effect on sleep. There are numerous examples of compounds lacking central efficacy due to insufficient brain exposure. For example, the reduced ability of second-generation H1 anti-histaminic drugs to cross the blood-brain barrier (BBB) as compared to the first generation of drugs, prevents them from causing centrally-mediated side effects such as sedation [48,49,50]. Similarly, the antidiarrheal medication loperamide is a potent agonist of the μ opiate receptor that is devoid of opioid central effects at usual doses in patients [51]. This directly results from the

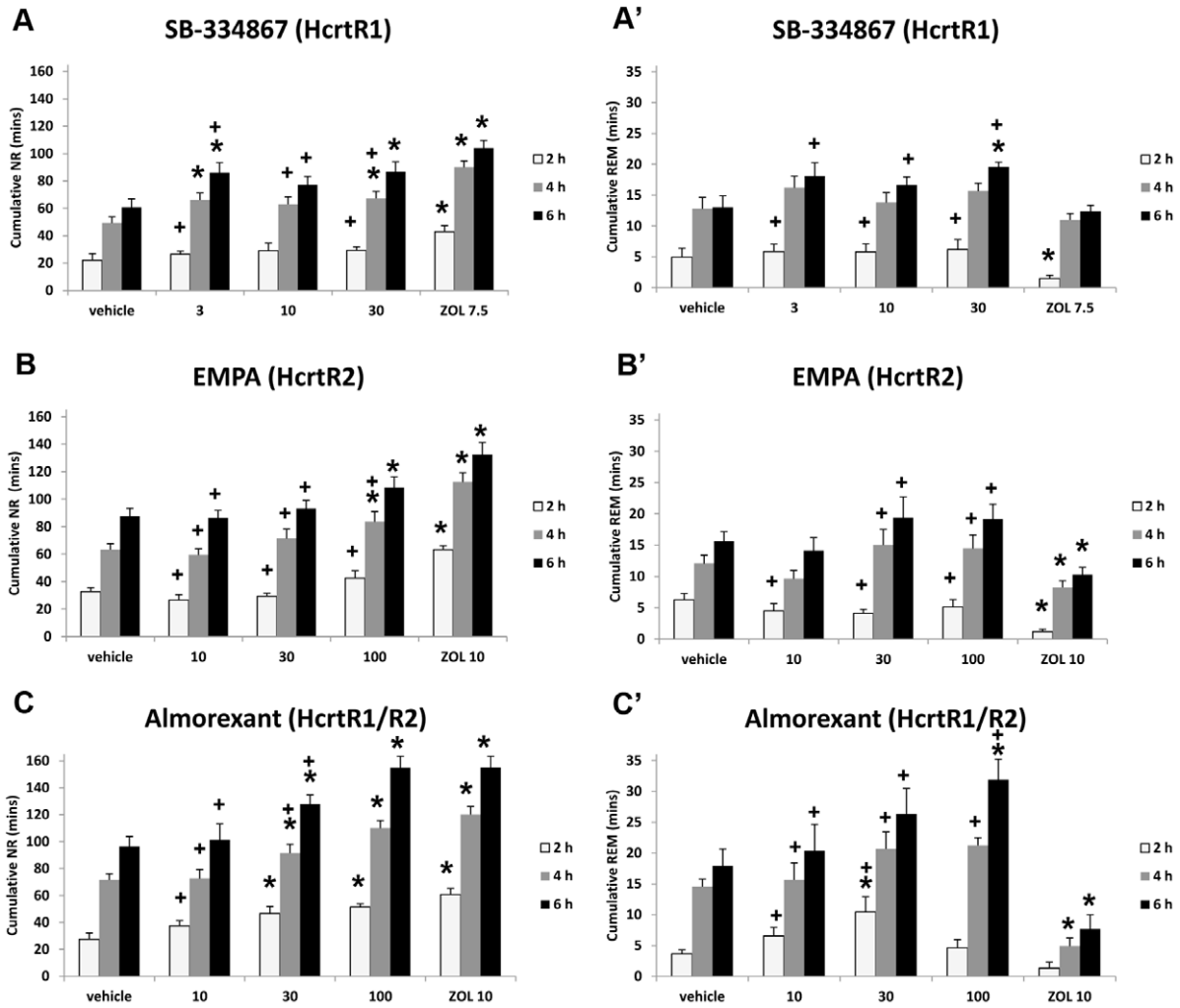

Figure 4. Cumulative time in NR and REM sleep over the first 2, 4 and 6 h following drug administration. (A–C) Cumulative time spent in NR sleep following SB-334867 (**A**), EMPA (**B**) and almorexant **(C)** compared to zolpidem (ZOL). **(A'–C')** Cumulative time spent in REM sleep for the same drug treatments. (One-way repeated measures ANOVA followed by paired two-tail *t* tests; n = 8 per group). Data represent the mean±SEM. *, significantly different from vehicle; **+**, significantly different from ZOL.
doi:10.1371/journal.pone.0039131.g004

Table 3. REM:NR ratios for the 6 h period following the administration of SB-334867, EMPA and almorexant.

Vehicle	**SB-334867**	**SB-334867**	**SB-334867**	**ZOL**
	3 mg/kg	**10 mg/kg**	**30 mg/kg**	**7.5 mg/kg**
0.22±0.039	0.21±0.016^{+}	0.22±0.023^{+}	0.23±0.016^{+}	0.12±0.009*
Vehicle	**EMPA**	**EMPA**	**EMPA**	**ZOL**
	10 mg/kg	**30 mg/kg**	**100 mg/kg**	**10 mg/kg**
0.18±0.018	0.16±0.019^{+}	0.21±0.032^{+}	0.18±0.027^{+}	0.08±0.008*
vehicle	**Almorexant**	**Almorexant**	**Almorexant**	**ZOL**
	10 mg/kg	**30 mg/kg**	**100 mg/kg**	**10 mg/kg**
0.18±0.020	0.19±0.026^{+}	0.20±0.022^{+}	0.21±0.021^{+}	0.05±0.013*

* = significantly different from vehicle ($p<0.05$), $^{+}$ = significantly different from ZOL ($p<0.05$).
doi:10.1371/journal.pone.0039131.t003

low brain exposure caused by the P-glycoprotein (P-gp) transporter at the BBB [51]. Administration of the drug to P-gp-deficient mice or co-administration with a P-gp blocker both increase brain levels and trigger central effects typically observed with brain penetrant opioids, such as analgesia [52,53] or respiratory depression [54]. Our observation made with SB-408124 underscores that verification of brain penetration is a prerequisite for the conception and use of centrally-acting drugs [55,56].

On the other hand, it is difficult to reconcile the poor brain penetration of SB-408124, both documented here and also evident in the study of Dugovic *et al.* (estimation: 0.012), with some indications of central localization following subcutaneous administration of 30 mg/kg, i.e. the 90% HCRTR1 occupancy observed in the *tenia tecta* and the SB-408124-mediated elevation of extracellular dopamine levels in the prefrontal cortex [32]. A heterogeneous distribution of the drug is unlikely, and further experiments will be necessary to delineate more precisely the free concentration of the compound, such as microdialysis studies and measures of binding to brain tissue homogenates.

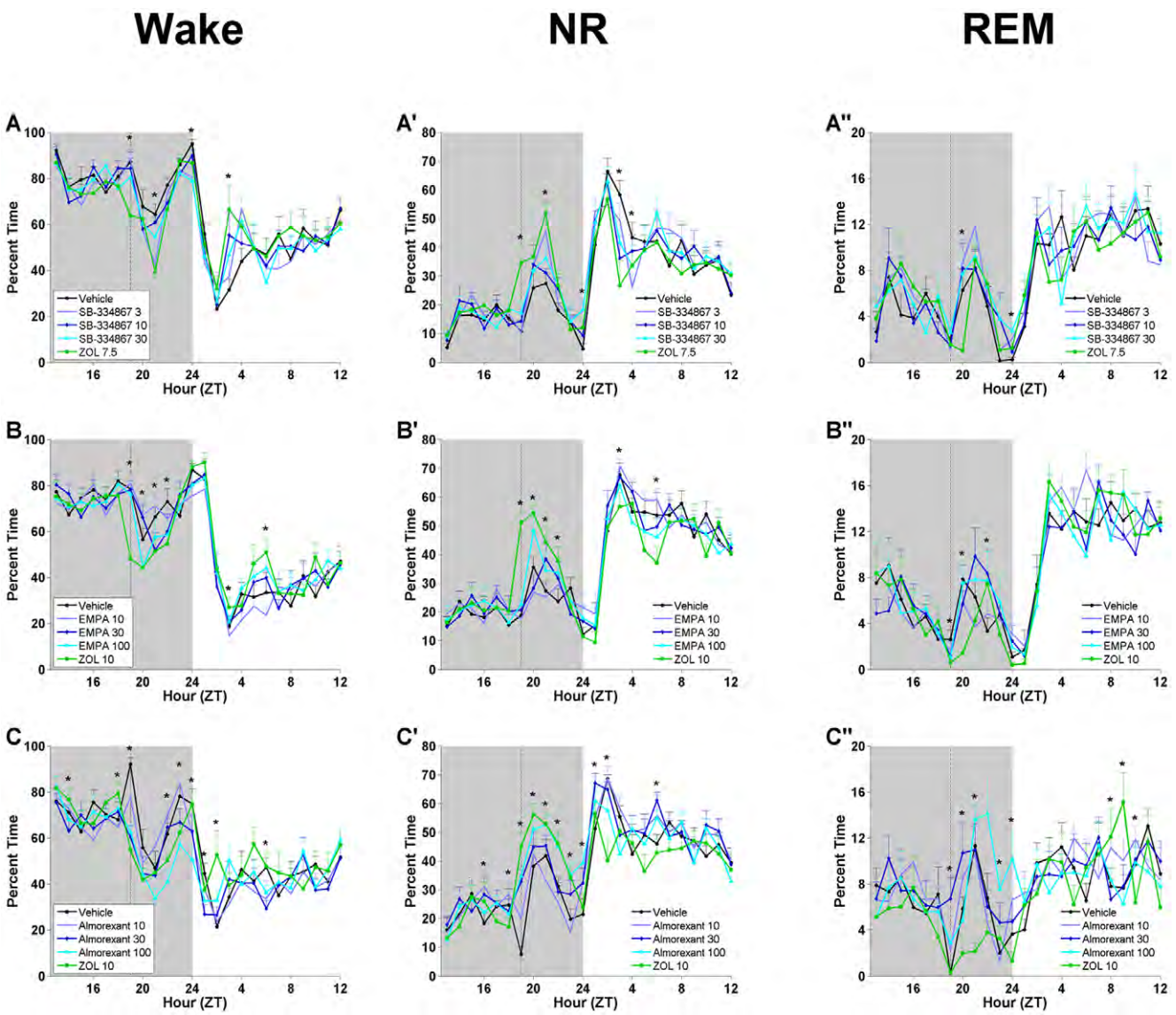

Figure 5. Hourly distribution of W, NR and REM sleep. W, NR and REM sleep for 6 h prior to and 18 h after administration of SB-334867 **(A)**, EMPA **(B)**, and almorexant **(C)** as compared to zolpidem (ZOL) and vehicle. Shaded area represents the dark phase; vertical dotted line in each panel indicates the time of injection. **(A)** Hourly amounts of wakefulness following SB 334867. **(A')** Hourly amounts of NR sleep following SB 334867. **(A'')** Hourly amounts of REM sleep following SB 334867. **(B)** Hourly amounts of wakefulness following EMPA. **(B')** Hourly amounts of NR sleep following EMPA. **(B'')** Hourly amounts of REM sleep following EMPA. **(C)** Hourly amounts of wakefulness following almorexant. **(C')** Hourly amounts of NR sleep following almorexant. **(C'')** Hourly amounts of REM sleep following almorexant. Data represent the mean±SEM (n = 8 rats per group). *, $p<0.05$. For detailed statistical results, see Text S1.
doi:10.1371/journal.pone.0039131.g005

Dual HCRTR Antagonists as Potential Hypnotic Medications

Dual HCRTR1/R2 antagonists are now well-established to induce sleep. In rats, almorexant administered po at the beginning of the dark phase promoted both NR and REM sleep and, at a higher dose, reduced NR and REM latency [27]. The effects on sleep duration but not sleep latency were confirmed when almorexant was administered sc [32]. Here, we report that almorexant given ip at the mid-dark phase also increases sleep duration. However, in contrast to Dugovic *et al.*, we found that almorexant at 30 and 100 mg/kg reduced NR latency and the 30 mg/kg dose also decreased REM latency. These differences likely reflect the greater sensitivity of the sleep/wake bioassay when injections occur in the mid-dark period after a sleep debt has accumulated. Recently, other dual HCRTR1/R2 antagonists have also been reported to reduce active wake and increase both NR or delta sleep and REM sleep when administered near the mid-dark phase [25,26,27,28,29,30,31,57]. Thus, multiple HCRTR1/R2 antagonists seem to be effective in inducing sleep.

Our results indicate some promising aspects of HCRT antagonists as hypnotic agents. First, in contrast to current hypnotics such as zolpidem which increase NR and suppress REM sleep, none of the three HCRTR antagonists affected the REM:NR ratio, indicating that both REM and NR increased proportionally. Second, in comparison to zolpidem, HCRTR antagonists only triggered a limited, physiological reduction of body temperature. Lastly, no excess wakefulness was observed during the subsequent light period. A proportional increase of

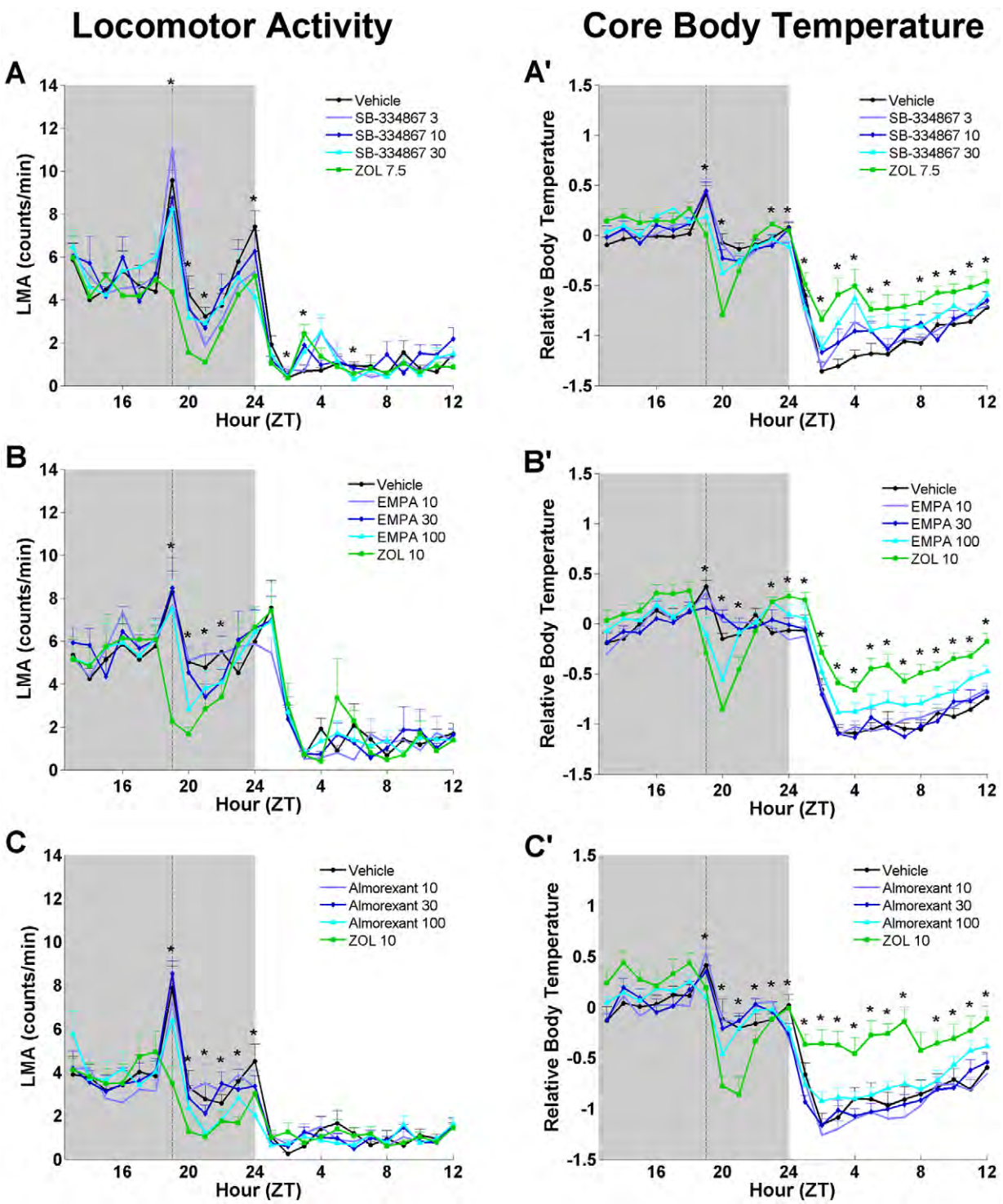

Figure 6. Average hourly LMA and relative T_{core}. LMA and relative T_{core} for 6 h prior to and 18 h after administration of SB-334867 (A), EMPA (B), and almorexant (C) as compared to zolpidem (ZOL) and vehicle. Shaded area represents the dark phase; vertical dotted line in each panel indicates the time of injection. (A) Average hourly LMA following SB-334867. (A′) The average hourly T_{core} following SB-334867. (B) The average hourly LMA following EMPA. (B′) The average hourly T_{core} following EMPA. (C) The average hourly LMA following almorexant. (C′) The average hourly T_{core} following almorexant. Data represent the mean±SEM (n = 8 rats per group). *, $p<0.05$. For detailed statistical results see Text S1.
doi:10.1371/journal.pone.0039131.g006

REM and NR sleep without rebound wakefulness and a mild change in core temperature are desirable properties of substances that induce "physiological" sleep.

On the other hand, the mechanism by which these HCRTR antagonists increased sleep duration suggests disruption of normal sleep/wake architecture. SB-334867 increased NR through a

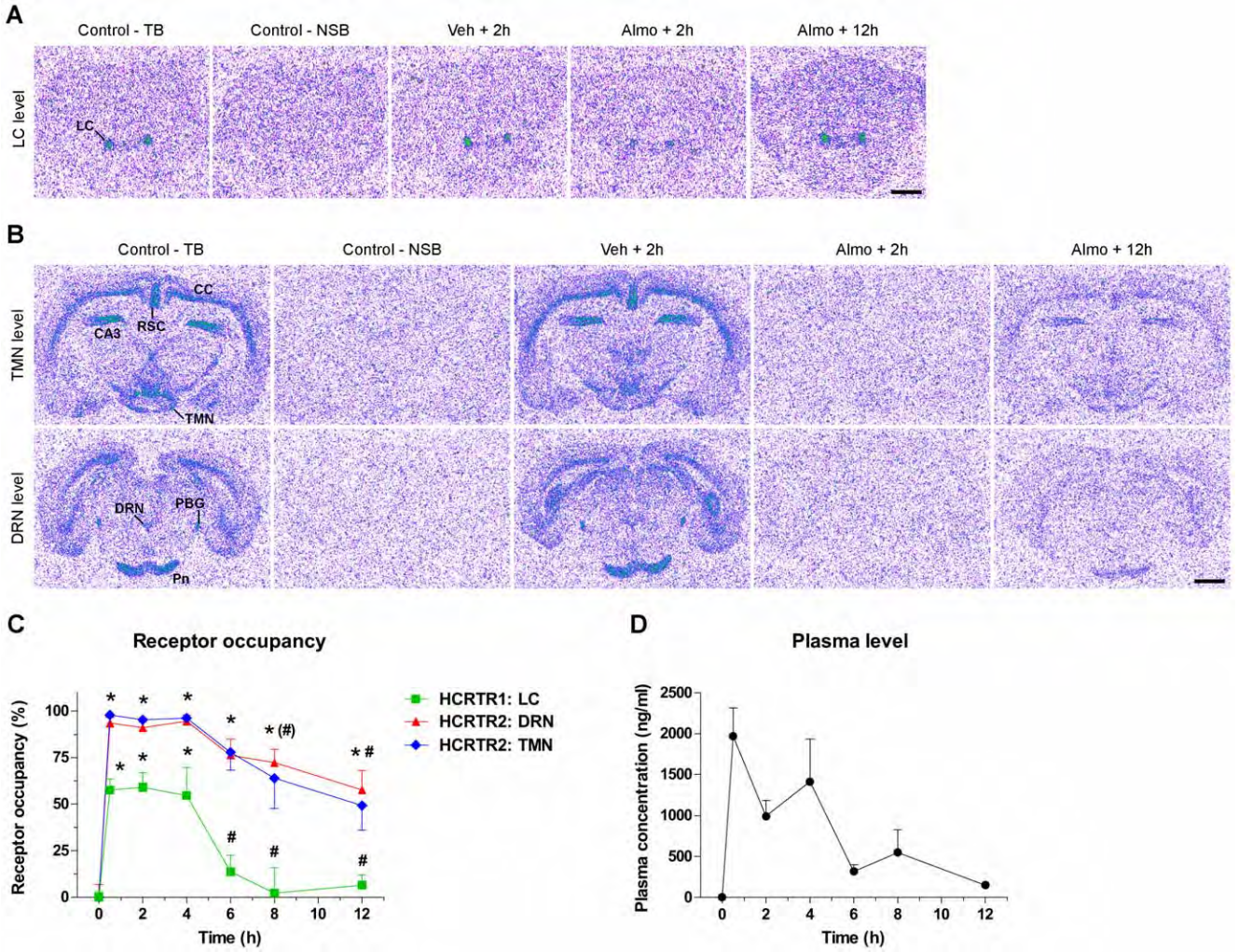

Figure 7. Time-course of HCRT1R and HCRT2R occupancies by almorexant. (A,B) Representative autoradiograms showing [3H]SB-674042 (5 nM) binding to HCRTR1 **(A)** and [3H]EMPA (1 nM) binding to HCRTR2 **(B)** in rat coronal brain sections. For both receptors, total binding (*TB*) was maximal in control animals (not injected) sampled at time 0 (*t0*). For HCRTR1 **(A)**, a clear signal was evident in the locus coeruleus (*LC*), which could be displaced by co-incubation with an excess of cold SB-674042 (10 µM) (non-specific binding, *NSB*). In contrast to vehicle administration (*Veh, 2 h*), almorexant (30 mg/kg injected intraperitoneally at ZT18) attenuated such specific signal after 2 h (*Almo, 2 h*), but not after 12 h (*Almo, 12 h*). For HCRTR2 **(B)**, signal was observed in various brain regions, including the tuberomammillary nuclei (*TMN*), cerebral cortex (*CC*), field CA3 of the hippocampus (*CA3*), retrosplenial cortex (*RSC*), dorsal raphe nuclei (*DRN*), pontine nuclei (*Pn*) and parabigeminal nuclei (*PBG*). [3H]EMPA could be displaced by co-incubation with an excess of Cp5 (10 µM) (*NSB*). HCRTR2 binding became minimal 2 h after almorexant (*Almo, 2 h*), but not after Vehicle (*Veh+2 h*), administration. After 12 h (*Almo, 12 h*), HCRTR2 binding was intermediate. Scale bars, 2 mm. **(C)** Time course of HCRTR1 and HCRTR2 occupancies by almorexant. Receptor occupancy was calculated by measuring the specific binding at various time points in the *LC* for HCRTR1, and in the *TMN* and *DRN* for HCRTR2. *, $p<0.001$ versus time 0; (#), $p<0.05$ (TMN only), #, $p<0.05$ (TMN) or $p<0.01$ (DRN), vs. time 30 min (one-way ANOVA followed by Dunnett's analysis). **(D)** Almorexant plasma concentrations. Data represent the mean±SEM (n = 5 rats per group).
doi:10.1371/journal.pone.0039131.g007

combination of small increases in both the number and duration of NR bouts that, although not significant for any particular hour, cumulatively summated into an overall significant NR increase at 4 and 6 h. For EMPA, a greater number of NR bouts underlie the overall increase in NR at the highest dose. For almorexant, NR augmentation resulted from an increased number of NR bouts without a change in bout duration, confirming previous results [32]. The increase in NR, however, was also associated with greater numbers of both W and REM bouts, particularly at the highest dose examined. Thus, although almorexant produces an overall increase in NR sleep that is greater than the other HCRTR antagonists, this is achieved through a fragmented sleep architecture. In this regard, almorexant-treated rats appear somewhat similar to *orexin* null mutant [4] or *orexin/ataxin-3* [12] mice which have disrupted sleep architecture (although these strains also exhibit cataplexy). However, the fragmentation of sleep architecture induced by dual HCRTR antagonists is consistent with the concept that the HCRT system stabilizes arousal states and minimizes the number of transitions between states [58]. Since drugs were administered to healthy animals during their active period, a more fragmented sleep architecture would be predicted. Rather than driving sleep *per se*, HCRTR antagonism seems to create a permissive neural environment for sleep to occur. Since the drive for sleep was low at the time of administration, more frequent sleep bouts without increases in bout durations could be expected.

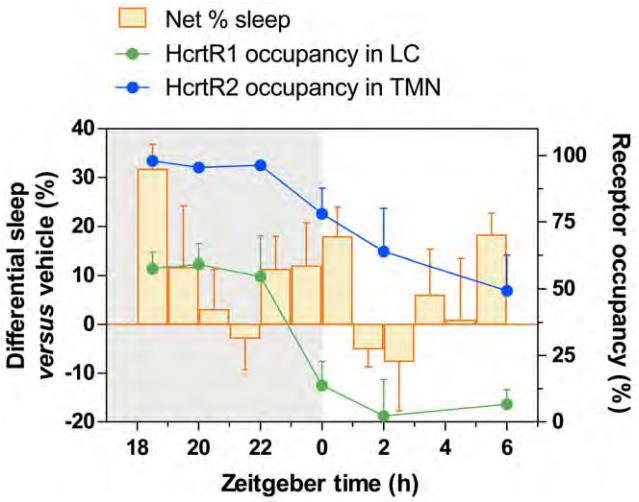

Figure 8. Net effect of almorexant on the percentage of sleep compared to HCRTR1 and HCRTR2 occupancies. The percentage of total sleep (%NR + %REM) in the vehicle-injected animals was subtracted from that of almorexant-treated rats (30 mg/kg) and was plotted over time. HCRTR1 occupancy in the locus coeruleus (*LC*) and HCRTR2 occupancy in the tuberomammillary nuclei (*TMN*) are shown in parallel. Injection occurred at ZT18. *Gray area,* dark phase; *White area,* light phase.
doi:10.1371/journal.pone.0039131.g008

Absence of Cataplexy but Facilitation of REM Sleep

One concern regarding the development of HCRTR antagonists is the possibility of inducing cataplexy as occurs in *HcrtR2* mutant dogs [3] or *HcrtR2* null mutant mice [59]. In the present study, we saw no evidence of cataplexy produced by any of the three compounds, even at the highest dose tested. However, almorexant significantly increased REM bout duration during the first hour after treatment and the highest dose – which presumably resulted in the most complete HCRTR blockade – produced 2 to 3 fold as many REM bouts during the latter half of the dark period when compared to vehicle. These observations indicate that HCRTR antagonism facilitates REM sleep occurrence, as noted by others [59].

Relationship between HCRTR Occupancy and Sleep

Whereas 30 mg/kg ip almorexant resulted in approximately 50% HCRTR1 occupancy, HCRTR2 occupancy was nearly complete in brain regions important for sleep/wake control. Moreover, while HCRTR1 occupancy declined after 4 h, HCRTR2 occupancy remained high even 12 h after treatment. While our results for HCRTR2 are consistent with a previous report, those for HCRTR1 differ [32]. A primary difference between these studies is the brain location used for determination of HCRTR1 occupancy: whereas Dugovic *et al.* used the *tenia tecta*, we measured HCRTR1 occupancy in the *LC*, an area implicated in sleep/wake control.

Figure 8 correlates RO with the net amount of sleep induced by almorexant at 30 mg/kg compared to vehicle. Since HCRTR2 occupancy is virtually 100% following this dose of almorexant while HCRTR1 occupancy is ~50%, it is likely that the stronger sleep-promoting effects observed at 100 mg/kg are due to greater HCRTR1 blockade. Figure 8 demonstrates that the sleep-promoting effects of almorexant do not simply mirror the RO data. The greatest amount of sleep occurred in the first hour after almorexant administration when occupancy of HCRTRs was maximal. Surprisingly, despite elevated occupancy of HCRTRs in subsequent hours, the hypnotic effect dissipated, suggesting that other arousal-promoting systems can overcome HCRTR blockade and produce wakefulness. In contrast, near the end of the dark phase when sleep pressure is elevated, partial HCRTR blockade was sufficient to produce sleep. These data highlight the contrasting sleep-promoting mechanisms between HCRTR antagonists and other hypnotic medications such as zolpidem. Whereas the latter compounds trigger long-lasting sleep and affect sleep intensity (sleep-inducing effect), HCRTR antagonists seem to merely antagonize wakefulness, generating conditions that allow sleep to occur (sleep permissive action).

Conclusion

Our results support the hypothesis that dual HCRTR1/R2 blockade is more effective in promoting sleep than selective blockade of either HCRTR alone. A similar conclusion was reached in a recent study of HCRT receptor knockout mice [45]. Although both HCRTR1 (SB-334867) and HCRTR2 (EMPA) antagonists produced somnogenic effects, neither promoted sleep to the levels of the dual HCRTR antagonist almorexant. Furthermore, since the lowest doses of almorexant that were sleep-promoting (30 mg/kg) bind virtually 100% of the HCRTR2s while only 50% of the HCRTR1s are occupied at that dose, the stronger sleep-promoting effects of higher doses are likely due to additional blockade of HCRTR1. These data support the notion that HCRTR antagonists are a promising avenue for sleep/wake therapeutics, with the qualifications stated above. However, given the involvement of the HCRT system in many physiological functions [9,60] including respiratory control [61,62,63,64], careful screening for side effects of HCRTR antagonists will be needed.

Supporting Information

Figure S1 Chemical structures of the compounds used in this study. Receptor selectivity is indicated into parentheses. All compounds except zolpidem are selective HCRTR antagonists. Zolpidem is a gama-aminobutyric acid (GABA) A-receptor agonist.
(TIF)

Figure S2 Hourly delta power normalized to the 24 h average vehicle control. A: 3 concentrations of SB-334867 vs. ZOL and vehicle. ANOVA is significant for treatment by time only ($F = 3.80$, $p<0.0001$). For treatment by time: **ZT19:** SB-334867 at 3 mg/kg > vehicle; ZOL >334867 at 3 and 10 mg/kg and vehicle. **ZT24:** 334867 at 3 and 10 mg/kg > ZOL; Vehicle >334867 at 10 and 30 mg/kg and ZOL **B:** 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA is significant for treatment (see legend, $F = 13.47$, $p<0.0001$) and for treatment by time ($F = 11.86$, $p<0.0001$). For treatment by time: **ZT19:** ZOL > all other conditions. **ZT20:** ZOL > all other conditions. **ZT21:** EMPA at 30 mg/kg > vehicle; ZOL > EMPA at 100 mg/kg and vehicle. **ZT22:** EMPA at 30 mg/kg > vehicle. **ZT23:** EMPA at 10 mg/kg > ZOL. **C:** 3 concentrations of almorexant vs. ZOL and vehicle. ANOVA is significant for treatment by time only ($F = 2.63$, $p = 0.0005$). For treatment by time: **ZT20:** Vehicle > almorexant at 100 mg/kg. **ZT23:** Almorexant at 10 mg/kg > vehicle. **ZT24:** Vehicle > almorexant at 100 mg/kg.
(TIF)

Figure S3 Hourly distribution of Wake Bout Duration and the Number of Wake Bouts. Wake Bout Duration (left) and Number of Wake Bouts (right) for 6 h prior to and 18 h after administration of SB-334867 (**A**), EMPA (**B**), and almorexant (**C**)

as compared to zolpidem (ZOL). Shaded area represents the dark phase; vertical dotted line shows the first h following injection. **A:** The Wake Bout Duration for 3 concentrations of SB 334867 vs. ZOL and vehicle. No significant differences were found. **A':** The Wake Bout Number for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT1-ZT6 is significant for treatment by time ($F = 1.82$, $p = 0.02341$). For treatment by time: **ZT2:** SB 334867 at 10 mg/kg and vehicle < ZOL vehicle < SB 334867 at 30 mg/kg **ZT4:** SB 334867 at 30 mg/kg and ZOL < vehicle **B:** The Wake Bout Duration for 3 concentrations of EMPA vs. ZOL and vehicle. No ANOVA's were significant. **B':** The Wake Bout Number for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 3.65$, $p = 0.01350$). ANOVA for ZT7-ZT12 is significant for treatment ($F = 4.24$, $p = 0.00647$) For treatment by time: **ZT19:** vehicle < ZOL **ZT20:** vehicle < EMPA at 30 mg/kg **ZT22:** vehicle < ZOL **ZT24:** vehicle < EMPA at 10, 30 and 100 mg/kg **ZT7:** EMPA at 10 mg/kg < ZOL **ZT11:** vehicle < ZOL **C:** The Wake Bout Duration for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 4.01$, $p = 0.01077$) and for treatment by time ($F = 2.32$, $p = 0.00234$). For treatment by time: **ZT20:** Almorexant at 100 mg/kg < ZOL **ZT21:** Almorexant at 30 and 100 mg/kg < ZOL **ZT22:** Almorexant at 100 mg/kg < ZOL and vehicle **C':** The Wake Bout Number for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 8.82$, $p = 0.00001$) and for treatment by time ($F = 2.07$, $p = 0.00769$). ANOVA for ZT7-ZT12 is significant for treatment ($F = 3.39$, $p = 0.02208$). For treatment by time: **ZT19:** vehicle < Almorexant at 30 and 100 mg/kg **ZT20:** ZOL < Almorexant at 10, 30 and 100 mg/kg **ZT21:** ZOL < Almorexant at 30 and 100 mg/kg **ZT22:**ZOL and vehicle < Almorexant at 100 mg/kg **ZT23:** vehicle < Almorexant at 100 mg/kg **ZT24:** vehicle < Almorexant at 100 mg/kg **ZT9:** Almorexant at 10 and 30 mg.kg < vehicle.
(TIF)

Figure S4 Hourly distribution of NR Bout Duration and Number of NR Bouts. NR Bout Duration (left) and Number of NR Bouts (right) for 6 h prior to and 18 h after administration of SB-334867 (**A**), EMPA (**B**), and almorexant (**C**) as compared to zolpidem (ZOL). Shaded area represents the dark phase; vertical dotted line shows the first h following injection. **A:** The NR Bout Duration for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 12.46$, $p<0.00001$) and for treatment by time ($F = 4.57$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.70$, $p = 0.00498$) and for treatment by time ($F = 3.16$, $p = 0.00004$). For treatment by time: **ZT19:** SB 334867 at 3 mg/kg and vehicle < ZOL **ZT20:** all other conditions < ZOL **ZT21:** vehicle < SB 334867 at 30 mg/kg and ZOL **ZT24:** vehicle < SB 334867 at 3 mg/kg **ZT1:** ZOL < SB 334867 at 3 and 10 mg/kg and vehicle SB 334867 at 3 mg/kg < vehicle **ZT3:** SB 334867 at 30 mg/kg and ZOL < vehicle **A':** The NR Bout Number for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT1-ZT6 is significant for treatment by time ($F = 1.81$, $p = 0.02532$). For treatment by time: **ZT1:** vehicle < SB 334867 at 3 and 30 mg/kg and ZOL **ZT4:** SB 334867 at 3 mg/kg < vehicle **B:** The NR Bout Duration for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 13.46$, $p<0.00001$) and for treatment by time ($F = 5.34$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 7.99$, $p = 0.00010$). ANOVA for ZT7-ZT12 is significant for treatment ($F = 3.03$, $p = 0.02981$). For treatment by time: **ZT19:** all other conditions < ZOL **ZT20:** all other conditions < ZOL **ZT23:** ZOL < EMPA at 10 mg/kg **ZT24:** ZOL < EMPA at 30 mg/kg **ZT2:** ZOL < EMPA at 30 mg/kg **ZT3:** ZOL < EMPA at 10 and 100 mg/kg and vehicle **ZT5:** ZOL < EMPA at 10 and 100 mg/kg and vehicle EMPA at 30 and 100 mg/kg < vehicle **ZT6:** ZOL < EMPA at 10 mg/kg and vehicle EMPA at 100 mg/kg < vehicle **B':** The NR Bout Number for 3 concentrations of EMPA vs. ZOL and vehicle. No ANOVA's were significant. **C:** The NR Bout Duration for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 16.44$, $p<0.00001$) and for treatment by time ($F = 5.34$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.83$, $p = 0.00433$) and for treatment by time ($F = 2.24$, $p = 0.00341$). For treatment by time: **ZT19:** all other conditions < ZOL vehicle < Almorexant at 100 mg/kg **ZT20:** all other conditions < ZOL **ZT21:** all other conditions < ZOL **ZT22:** Almorexant at 10 and 30 mg/kg < ZOL **ZT2:** ZOL < Almorexant at 10 and 30 mg/kg and vehicle Almorexant at 100 mg/kg < vehicle **ZT3:** ZOL < Almorexant at 10 mg/kg **ZT4:** ZOL < Almorexant at 10 mg/kg **ZT5:** ZOL < Almorexant at 10 mg/kg **ZT6:** ZOL < Almorexant at 30 mg/kg **C':** The NR Bout Number for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 12.58$, $p<0.00001$) and for treatment by time ($F = 2.41$, $p = 0.00149$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.18$, $p = 0.00890$). For treatment by time: **ZT19:** vehicle < Almorexant at 30 and 100 mg/kg **ZT20:** ZOL < Almorexant at 10, 30 and 100 mg/kg vehicle < Almorexant at 100 mg/kg **ZT21:** ZOL < Almorexant at 10, 30 and 100 mg/kg **ZT22:** ZOL and vehicle < Almorexant at 100 mg/kg **ZT23:** vehicle < Almorexant at 100 mg.kg **ZT24:** vehicle < Almorexant at 100 mg.kg **ZT1:** vehicle < ZOL.
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Figure S5 Hourly distribution of REM Sleep Bout Duration and the Number of REM Sleep Bouts. REM Sleep Bout Duration (left) and the Number of REM Sleep Bouts (right) for 6 h prior to and 18 h after administration of SB-334867 (**A**), EMPA (**B**), and almorexant (**C**) as compared to zolpidem (ZOL). Shaded area represents the dark phase; vertical dotted line shows the first h following injection. **A:** The REM Bout Duration for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 4.40$, $p = 0.00692$) and treatment by time ($F = 2.16$, $p = 0.00500$). For treatment by time: **ZT19:** ZOL < SB 334867 at 3 mg/kg **ZT20:** ZOL < all other conditions **ZT23:** vehicle < all other conditions **ZT24:** SB 334867 at 10 mg/kg < ZOL vehicle < SB 334867 at 3 mg/kg **A':** The REM Bout Number for 3 concentrations of SB 334867 vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment by time only ($F = 4.49$, $p = 0.00625$). For treatment by time: **ZT20:** ZOL < all other conditions **ZT24:** vehicle < SB 334867 at 30 mg/kg **B:** The REM Bout Duration for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment by time ($F = 1.71$, $p = 0.03515$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.88$, $p = 0.00015$) and for treatment by time ($F = 2.81$, $p = 0.00015$). For treatment by time: **ZT21:** ZOL < EMPA at 100 mg/kg **ZT24:** EMPA at 100 mg/kg < vehicle **ZT1:** EMPA at 100 mg/kg < ZOL all other conditions < vehicle **ZT4:** EMPA at 10 and 30 mg/kg < vehicle **ZT5:** ZOL < EMPA at 10 mg/kg **B':** The REM Bout Number for 3 concentrations of EMPA vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 3.99$, $p = 0.00888$) and for treatment by time ($F = 1.96$, $p = 0.01112$). For treatment by time: **ZT20:** ZOL < all other conditions **ZT22:** vehicle < ZOL **ZT23:** ZOL < vehicle **C:** The REM Bout Duration for 3 concentrations of Almorexant vs.

ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment by time ($F = 6.91$, $p<0.00001$). ANOVA for ZT1-ZT6 is significant for treatment ($F = 4.45$, $p = 0.00657$). For treatment by time: **ZT19:** ZOL and vehicle < Almorexant at 10, 30 and 100 mg/kg **ZT20:** all other conditions < ZOL **ZT24:**ZOL < Almorexant at 10 and 100 mg/kg and vehicle Almorexant at 30 mg/kg < vehicle **C':** The REM Bout Number for 3 concentrations of Almorexant vs. ZOL and vehicle. ANOVA for ZT19-ZT24 is significant for treatment ($F = 9.29$, $p = 0.00007$) and for treatment by time ($F = 2.96$, $p = 0.00010$). For treatment by time: **ZT19:** ZOL and vehicle < Almorexant at 30 mg/kg **ZT20:** ZOL < Almorexant at 10 and 30 mg/kg and vehicle **ZT21:** ZOL < all other conditions **ZT22:** ZOL and vehicle < Almorexant at 100 mg/kg **ZT23:** vehicle < Almorexant at 100 mg/kg **ZT24:** ZOL and vehicle < Almorexant at 100 mg/kg.
(TIF)

Figure S6 Brain concentration of almorexant. Time course of almorexant concentration in the brain of rats injected intraperitoneally with 30 mg/kg at the mid-dark phase (same animals as in Figures 7). Data are the mean±SEM (n = 5 rats per group).
(PDF)

Figure S7 HCRTR2 occupancy in the cerebral cortex, retrosplenial cortex, pontine nuclei, and hippocampus. Data are the mean±SEM (n = 5 rats per group). *, $p<0.001$ vs. time 0; ##, $p<0.01$, #, $p<0.05$ vs. time 30 min (one-way ANOVA followed by Dunnett's analysis). Almorexant plasma concentrations (data from Figure 7) are shown for comparison.
(TIF)

Materials and Methods S1 Expanded materials and methods for both *in vitro* and *in vivo* experiments as referenced in the text.
(DOCX)

Text S1 Expanded legends for Figures 5 and 6 that include detailed statistical results.
(DOCX)

Table S1 Pharmacokinetic assessment of almorexant, SB-334867 and SB-408124 after i.v. and p.o. administration to Wistar rat.
(DOCX)

Table S2 CEREP selectivity screen in the broad radioligand binding assays were undertaken to determine the pharmacological activity of SB-334867.
(DOCX)

Table S3 Measures of state consolidation for 6 h following the administration of SB-334867.
(DOCX)

Table S4 Measures of state consolidation for 6 h following the administration of EMPA.
(DOCX)

Table S5 Measures of state consolidation for 6 h following the administration of almorexant.
(DOCX)

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Author Contributions

Conceived and designed the experiments: SRM FGR JLM JGW TSK EB PM. Performed the experiments: SRM FGR DV EB. Analyzed the data: SRM FGR DV JLM TSK EB PM. Wrote the paper: SRM FGR TSK.

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